İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DETECTION AND ENUMERATION OF ACETOCLASTIC METHANOGENS IN ANAEROBIC BIOREACTORS BY QUANTITATIVE FLUORESCENT *IN SITU* HYBRIDISATION TECHNIQUE

M.Sc. Thesis by

Meriç BATIOĞLU, B.Sc.

Department : Environmental Engineering

Programme: Environmental Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANAEROBİK BİYOREAKTÖRLERDEKİ ASETOKLASTİK METANOJENLERİN KANTİTATİF FLORESANLI YERİNDE HİBRİTLEŞME TEKNİĞİ İLE SAPTANMASI VE SAYIMI

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ŞUBAT 2006

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ÖNSÖZ

It is the mark of an instructed mind to rest easy with the degree of precision, which the nature of the subject permits, and not to seek exactness where only an approximation of the truth is possible.

Aristotle, 384-322 BC

Bu yüksek lisans tezi Environmental Engineering Group, School of Civil Engineering and Geosciences, University of Newcastle upon Tyne, İngilterede Şubat 2005 ile Ağustos 2005 tarihleri arasında sürdürülmüş literatür araştırma ve laboratuvar çalışmaları sonucunda ortaya çıkmıştır.

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Şubat, 2006 Meriç BATIOĞLU

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ABBREVIATIONS

CCP	Critical Control Point
COD	Chemical Oxygen Demand
CSLM	Confocal Scanning Laser Microscope
Cy 3	Indocarbocyanine
Cy 5	Indodicarbocyanine
DAPI	4', 6 diamidino-2-phenylindole
DGGE	Denaturant Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescent *In Situ* Hybridisation
FITC	Fluorescein isothiocyanate
MilliQ Water	Double Sterilised Water; autoclaved and filter sterilised
MPN	Most Probable Number
OLR	Organic Loading Rate
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
SQRT	Square Root Transformation
STW	Sewage Treatment Works
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
TGGE	Temperature Gradient Gel Electrophoresis
TOC	Total Organic Carbon
VFA	Volatile Fatty Acid

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K_m : Dissociation rate constant
K : Substrate concentration giving one-half the maximum growth rate
μ_{max} : Maximum growth rate

DETECTION AND ENUMERATION OF ACETOCLASTIC METHANOGENS IN ANAEROBIC BIOREACTORS BY QUANTITATIVE FLUORESCENT *IN SITU* HYBRIDISATION TECHNIQUE

SUMMARY

The fluorescent *in situ* hybridisation technique with 16S rRNA targeted oligonucleotide probes was used to detect and enumerate the microorganisms in the samples taken from a full-scale conventional single stage anerobic digester at Hexham Sewage Treatment Works (United Kingdom) and a lab-scale anaerobic membrane bioreactor that had been seeded with the mesophilic Hexham sludge. Each sample was hybridised with fluorescent oligonucleotide probes for *Bacteria, Archaea* and the defined group of methanogens namely, acetoclastic methanogens. Specific cell counts were determined by a combination of in situ hybridization and confocal laser scanning microscopy for statistical analysis.

The results of quantitative fluorescent in situ hybridisation (FISH) procedure indicated that the concentration values of *Methanoseate* and *Methanosarcina* are very close to each other for the sample taken from full-scale anaerobic digester. Besides, *Methanoseate* concentration is much higher than *Methanosarcina* concentration in the sample of lab-scale anaerobic membrane bioreactor. In addition, it has been determined that the archaeal cell concentrations are higher than the eubacterial cell concentrations for both of the samples.

The outcomes of analytical process showed that the variances of archaeal cells are homogenous for three dual hybridisations carried out for each sample in this study. Furthermore, the distribution types were determined as poisson distribution for *Archaea, Methanoseate* and *Eubacteria* and as negative binominal distribution for *Methanosarcina,* respectively. The statistical differences between the samples of the full-scale and the lab-scale anaerobic digesters were determined by means of i) plotting frequency distribution curves, ii) checking the normality with Anderson-Darling tests, iii)transforming the cell concentration for normal distribution according to the Box-Cox plots and iv) using one-way ANOVA (analysis of variance).

ANAEROBİK BİYOREAKTÖRLERDEKİ ASETOKLASTİK METANOJENLERİN KANTİTATİF FLORESANLI YERİNDE HİBRİTLEŞME TEKNİĞİ İLE SAPTANMASI VE SAYIMI

ÖZET

Floresanlı yerinde hibritleşme tekniği, 16S rRNA hedefli oligonukleotid problar ile Hexham Kanalizasyon Arıtım İşlerindeki (Birleşik Krallık) tam ölçekli konvensiyonel anaerobik biyoreaktörden ve mezofilik Hexham çamuru ile inokule edilmiş laboratuvar ölçekli anaerobik membran biyoreaktörden alınan örneklerdeki mikroorganizmaların saptanması ve sayımı için kullanılmıştır. Her iki örnek; bakteri, arke ve asetoklastik metanojenlere spesifik floresanlı oligonukleotid problar ile hibritlenmiştir. İstatistiksel analiz için yerinde hibritleşme ve konfokal lazer tarama mikroskobisi kombine edilerek spesifik hücre sayımları yapılmıştır.

Kantitatif FISH prosedürünün sonuçları, tam ölçekli anaerobik reaktörden alınan örnek için, *Methanoseate* ve *Methanosarcina* konsantrasyon değerlerinin birbirine çok yakın olduğunu göstermiştir. Laboratuvar ölçekli reaktör örneği için ise *Methanoseate* konsantrasyonu, *Methanosarcina* konsantrasyonundan çok daha yüksektir. Bunun yanında, her iki örnek içinde arkeal hücre konsantrasyonun, bakteriyel hücre konsantrasyonundan daha yüksek olduğu belirlenmiştir.

Analitik sürecin sonuçları, bu çalışmadaki her bir örnek için yürütülen üç farklı dual hibritleşmede de arkeal hücre değişkenliklerinin homojen olduğunu göstermiştir. Ayrıca, dağılım tipleri *Archaea*, *Methanoseate* ve *Eubacteria* için poisson dağılım; *Methanoarcina* için ise negatif binominal dağılım olarak belirlenmiştir. Ayrıca, i) sıklık dağılım eğrileri çizilerek, ii) Anderson-Darling testleriyle normalite kontrol edilerek, iii) Box-cox çizimlerine göre normal dağılım için gerekli olan hücre konsantrasyonu dönüşümleri yapılarak ve iv) değişkenlik analizi kullanılarak; tam ve laboratuvar ölçekli anaerobik reaktörlerden alınan örneklerin istatistiksel açıdan birbirlerinden farklı oldukları belirlenmiştir.

CHAPTER 1. INTRODUCTION

Anaerobic digestion is recognised as a promising biological treatment process due to many advantages over the more conventional aerobic processes including low levels of excess sludge production, low space requirements and the production of valuable biogas (Lettinga, 1995). However, some conditions should be met to enable an effective anaerobic reactor system such as a) high retention time of viable sludge, b) sufficient contact between viable bacterial biomass and wastewater, c) high reaction rates and absence of serious transport limitations d) acclimatized viable biomass and e) favourable environmental conditions.

Since the end of 19th century, anaerobic wastewater treatment systems have been used. The system was initially applied in decay of human excreta in air-tight earth closets. The developments in engineering systems led to the use of uncontrolled septic tanks and subsequently, temperature controlled completely - mixed bioreactors. Anaerobic microorganisms exhibit slow growth rate in biological treatment systems. Therefore, a number of novel anaerobic reactor configurations have been developed in order to achieve high treatment efficiency and reliability associated with a long sludge retention time (SRT) for industrial wastewaters. However, conventional or completely mixed anaerobic digester could also enable efficient sludge treatment.

Besides, several types of anaerobic bioreactor processes coupled with membranes have been studied for treating different wastewaters. Anderson *et al.* (1986) proved that high COD removal of over 98% and very low suspended solids in the effluent were possible with a membrane bioreactor including a porous membrane for biomass retention. Choo and Lee (1996) also reported that enhanced COD removal could be achieved using a membrane-coupled anaerobic bioreactor for treating alcohol-distillery wastewater. These studies indicated that the membrane unit could be linked with an anaerobic bioreactor system to obtain high COD removal efficiency for high strength organic industrial wastewaters, such as brewery and alcohol-distillery wastewater.

Anaerobic microbial consortia taking place within anaerobic digesters are responsible for the biochemical transformations of organic pollutants to methane. Two-thirds or

more of the methane produced in an anaerobic bioreactor is derived from acetate (Zinder, 1993). Of the many methanogenic genera, only two, *Methanosaeta* (formerly *Methanothrix*) and *Methanosarcina*, are known to grow by an acetoclastic reaction, producing methane from acetate (Zinder, 1993). *Methanosaeta* spp. are filamentous organisms which are known to grow only on acetate (Jetten *et al*., 1992). *Methanosarcina* spp. grow either as single coccoidal cells or in large clumps up to 1 to 3 mm in diameter. Besides acetate, *Methanosarcina* spp. are also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than do *Methanosarcina* spp., but their affinity for acetate is 5 to 10 times higher (Zinder, 1990; Jetten *et al*.,1992).

The understanding of the microbial ecology of the sludge present in the anaerobic digesters plays an important role in the controlling of startup and operational conditions. However, the ecology of microbial populations and communities in natural and engineered anaerobic systems remains largely unexplored (Raskin et al., 1994a). The lack of studies arises from the limitations of traditional identification and enumeration techniques, such as selective enrichment, pure-culture isolation, most-probable-number estimates, and determinative identification schemes.

Culture-independent molecular approaches are tending to replace culture-based methods for comparing the composition, diversity, and structure of microbial communities. Investigations based on these approaches have led to the conclusion that traditional methods of culturing natural populations have seriously underestimated archaeal and bacterial diversity (Bull *et al*., 2000). The inability to cultivate even the most numerous microorganisms from natural habitats has been referred to as the "great plate count anomaly" (Staley and Konopka, 1985).

The use of modern molecular techniques is essential for a better understanding of the ecophysiology. Therefore, hybridization assays using fluorescent rRNA targeted oligonucleotide probes became a promising tool for the study of the microbial populations (Amann *et al*., 1995). In several studies rRNA probes were applied to investigate the microbial population structure in activated sludge (Wagner *et al*., 1993; Manz *et al*., 1994; *Manz et al*., 1998) and methanogenic anaerobic reactors (Raskin *et al*., 1994).

Fluorescence *in situ* hybridization (FISH), the assay of choice for localization of specific nucleic acids sequences in native context, is a 20-year-old technology that

has been developed continuously (Levsky and Singer, 2003). This technique is common due to its different application ranges and significant advantages in reference to implementation and performance of *in situ* studies. De long *et al.* (1989) first applied whole-cell *in situ* hybridisation with fluorescently-labeled oligonucleotide probes to the microbial ecology studies. Since then, this technique has become the method of choice for reliable and rapid identification of microorganisms in environmental and medical samples (Wagner *et al*., 2003). However, there exist some methodological limitations such as cell permeability problems, target site accessibility, target site specificity and sensitivity in FISH technique (Head et al., 1998). These limitations could be overcome by increasing the sensitivity of FISH. Probes can be made more sensitive by one or a combination of the following approaches: (i) indirect labeling; (ii) the use of alternative, more sensitive labels; or (iii) multiple labeling (Amann et al., 1995). Instrumentation used for image acquisition also affects the overall sensitivity of FISH. Key methodological advances have allowed facile preparation of low-noise hybridization probes, and technological breakthroughs now permit multi-target visualization and quantitative analysis (Levsky and Singer, 2003).

In this study, all bacterial and archaeal populations; particularly, acetoclastic methanogens capable of producing methane from acetate present in both full-scale and lab-scale anaerobic digesters were detected and enumerated by means of quantitative FISH. The technique used along the experiments includes methodological advances such as the use of probes with more sensitive fluorescent dyes and technological breakthroughs such as confocal laser scanning microscopy.

1.1. Aim and Scope

The localisation and abundance of certain microorganisms are able to give very valuable information about reactor operating conditions. Furthermore, the combined use of different fluorescent dyes offers very interesting possibilities to study the ecology of certain strains within a community of related organisms (Elferink *et. al*, 1998).

The aim of this study is to detect and quantify the acetoclastic methanogens present in the anaerobic sludge samples taken from a conventional single stage anaerobic digester at Hexham Sewage Treatment Works and from a lab-scale anaerobic membrane bioreactor treating brewery wastewater seeded with Hexham sewage

sludge by using quantitative fluorescent *in situ* hybridisation. The ecology of *Bacteria* and *Archaea* including acetoclastic methanogens was tried to understand by dual hybridisation procedures with combination of confocal scanning microscopy. Once detected and quantified the populations of *Bacteria*, *Archaea* and particularly acetoclastic methanogens within the anaerobic digesters; statistical approach was applied to determine the variances within and between the samples. Furthermore, the reactor performances of full-scale and lab-scale anaerobic digesters were compared according to the predominance of *Methanoseate* or *Methanosarcina* depending on the methanogen kinetics.

CHAPTER 2. LITERATURE REVIEW

2.1. Process Description and Microbiology

Anaerobic digestion consists of a series of microbiological processes that convert organic compounds to methane and carbon dioxide, and reduce the volatile solids by 35% to 60%, depending on the operating conditions (U.S. EPA, 1992). The methanogenic conversion of organic matter to biogas (50–80% methane, 20–50% carbon dioxide, trace amounts of ammonia, hydrogen sulphide, hydrogen) under anaerobic conditions, in the absence of efficient terminal electron acceptors, such as sulphate or nitrate, is an intrinsic characteristic of all described ecosystems. The conversion process, known as anaerobic digestion, is mediated by complex microbial communities, with complete degradation to methane requiring the co-operative and sequential action of a number of different bacterial trophic groups (McHugh *et al*., 2003). Carbon flow to methane in anaerobic digesters with the microorganisms responsible for each step was depicted in Figure 2.1.

Microbial community can be divided into four main categories in the transformation of organic pollutants into simple compounds such as methane and carbondioxide: i) hydrolytic bacteria, ii) fermentative acidogenic bacteria, iii) acetogenic bacteria including the obligate hydrogen producing acetogens (OHPA) and homoacetogens, iv) methanogens including acetoclastic methaogens and hydrogen-utilising bacteria. There exists an interactive relationship between these microbial groups.

Hydrolytic bacteria are responsible for hydrolysis that is the first essential step in the degradation of complex polymers. Hydrolytic genera such as *Clostridium*, *Peptococcus*, *Vibrio*, *Micrococcus* and *Bacillus* produce extracellular hydrolytic enzymes to break down complex organic molecules into soluble monomer molecules. These enzymes are protease, lipase, cellulase, pectinase, amylase and chitinase. Anaerobic digesters contain between 10^8- 10^9 hydrolytic bacteria per ml comprising both facultative and obligate anaerobes (Anderson *et al*., 2003).

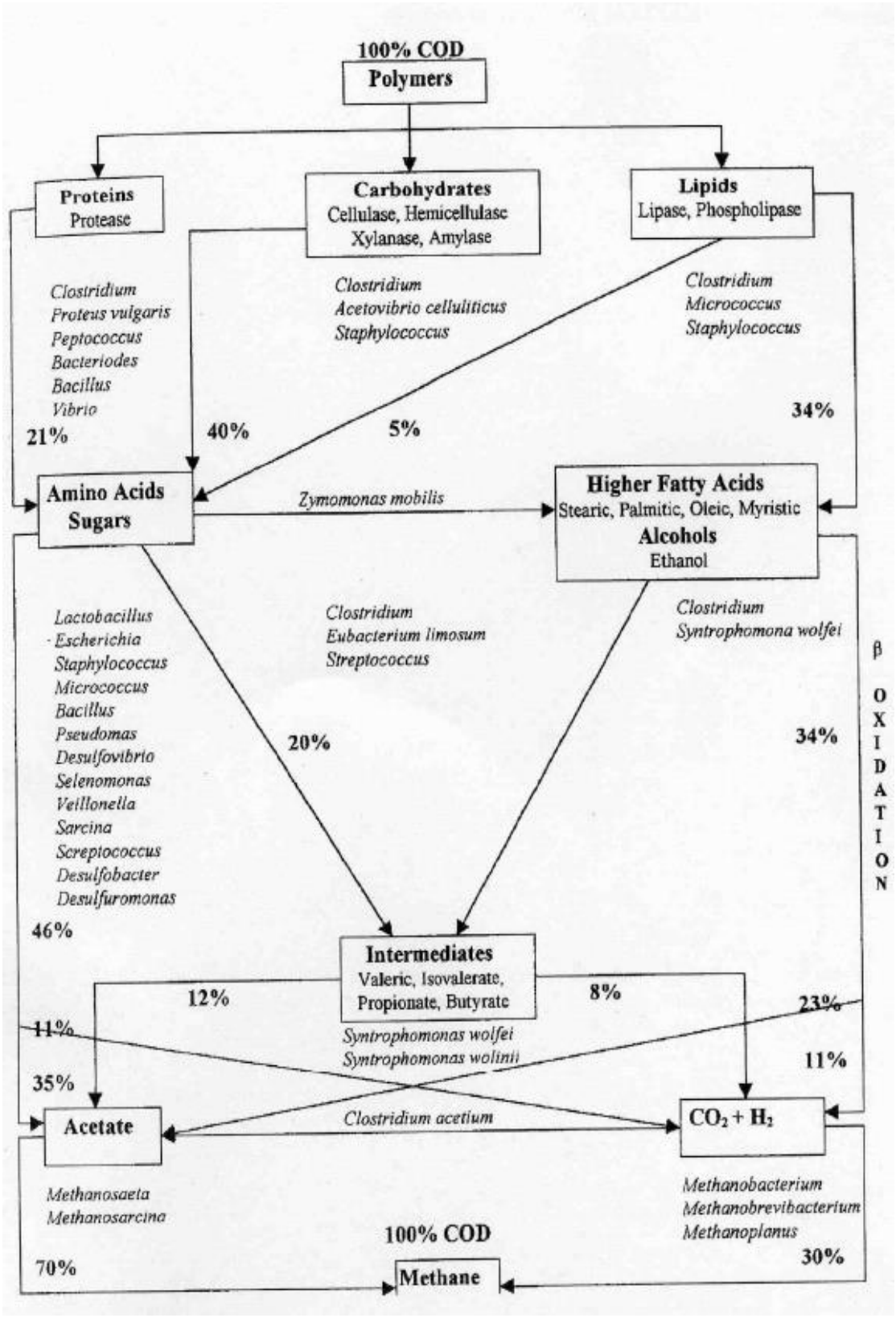


Figure 2.1. Carbon flow to methane in anaerobic digesters with the microorganisms responsible for each step (Adapted from Gujer and Zehnder, 1983; Cited in Anderson *et. al*, 2003).

Fermentative acidogenic bacteria convert sugar, amino acids and fatty acids to organic acids (acetic, propionic, formic, lactic, butyric or succinic acids), alcohols and ketones (ethanol, methanol, glycerol, acetone), CO_2 and H_2. There exists many different fermentative genera and species in acidogenesis process. These are *Clostridium, Bacteriodes, Ruminococcus, Butyribacterium, Propionibacterium, Eubacterium, Lactobacillus, Streptococcus, Pseudomonas, Desulfobacter, Micrococcus, Bacillus* and *Escherichia.* The number of acid forming bacteria per ml is in the range of 10^6-10^8 in anaerobic digesters (Archer and Kirsop, 1990).

Acetogenic bacteria are capable of producing acetate, carbon dioxide and hydrogen. These substrates are used by methanogens in the final stage of anaerobic digestion. Acetogens can be divided into two distinct groups depending on their metabolism. First group of acetogenic bacteria is the obligate hydrogen-producing acetogens (OHPA) and the second group of acetogens are the homoacetogens. OHPA such as *Syntrophomonas wolfei* and *Syntrophobacter wolinii* degrade the major fatty acid intermediates (propionate, butyrate), alcohols and other higher fatty acids. Mesophilic sludges contain approximately 4.5×10^6 *S. wolfei* per gram of digester sludge (Toerien and Hattingh, 1969). There exists a syntrophic relationship between OHPA and hydrogen-removing species such as methanogens and sulphate-reducing bacteria. Fatty acids inhibit the methanogens however, these methanogen inhibitors are degraded by OHPA. In addition, hydrogen inhibits OHPA, however existence of methanogens prevents this inhibitory effect by using hydrogen. The homoacetogens have the ability of catalysing acetate production from hydrogen and carbon dioxide. However, the number of this group is low indicating that their role is minor in anaerobic digesters.

The methanogens produce methane gas as the end-product of their metabolism. They belong to a seperate domain, the *Archaea*, and differ from procaryotes with their properties such as lacking muramic acid in the cell wall, presence of fluorescent spesific coenzyme F_{420} and F_{430}, and different rRNA sequences. Methanogens are divided into two groups depending on their substrate spesicificity. The first group is acetoclastic methanogens and the second group is hydrogen-utilising methanogens. The number of methanogens taking place in anaerobic digesters is in the range of 10^6 - 10^8 per ml (Toerien and Hattingh, 1969).

Acetate is known as the most important precursor of methane production. 70% of methane is produced from acetate through acetoclastic methanogens including *Methanoseate* (formerly *Methanothrix)* and *Methanosarcina*.

The genera of *Methanoseate* and *Methanosarcina* belong to two different families namely, *Methanosarcinaceae* and *Methanosaetaceae* taking place in the order of *Methanosarcinales*, as also seen in Figure 2.2.

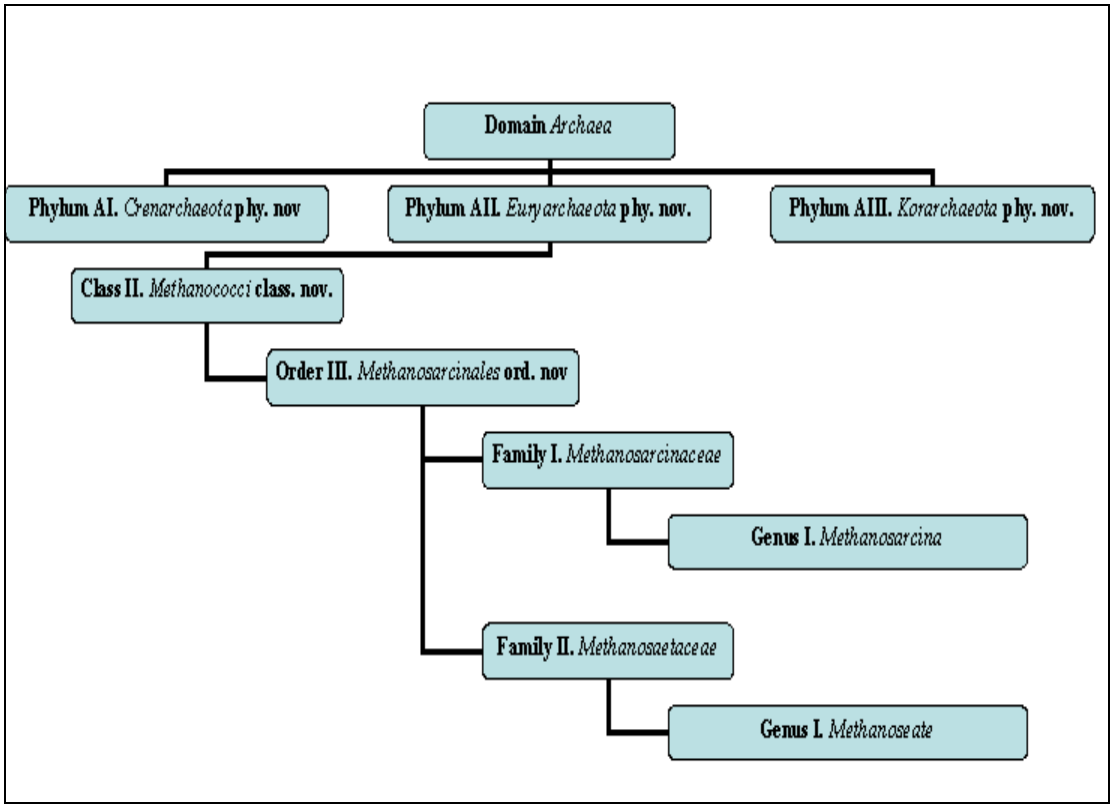


Figure 2.2. Taxonomic scheme of acetoclastic methanogens in the *Archaea* domain. (Data from Garrity and Holt, 2000).

The structure of *Methanoseate* is straight rods with flat ends; single cells are usually 0.8-1.3 μm wide by 2.0-7.0 μm long and are enclosed within a tubular sheath structure. The optimum temperature of this obligate anaerobe and Gram negative genus is 35-40°C with a range of 10-45°C for mesophilic strains; 55-60°C with a range of 30-70°C for thermophilic strains. Optimum pH is 6.5-7.5 with a range of 5.5-8.4 (Patel and Sprott, 1990).

Acetate is the only substrate that supports growth and methane production. Formate, H_2/CO_2, methanol and methylamines do not serve as substrates for growth or methane production. The F_{420} contents in *Methanoseate* species vary, and they are much lower than those reported in other methanogen species (Kamagata and Mikami,

1991), which may help to explain the weak to no observed autofluorescence when cells are excited with UV light at 350 nm wavelength (Patel, 1984; Zinder et al., 1987).

There exists a mesophilic and a thermophilic species belonging to the genus *Methanoseate* namely, *Methanoseate concilii* and *Methanoseate thermophila*. *M. concilii* survives in the range of mesophilic temperature (>10 to ≤45) and in the pH interval of ≤6.6 to >7.8. Optimal growth temperature and pH are 35-40°C and 7.1-7.5, respectively. *M. thermophila* survives in the range of thermophilic temperature (>30 to ≤70) and in the pH interval of >5.5 to ≤8.4. Optimal growth temperature and pH are 55-60°C and 6.5-6.7, respectively.

The structure of *Methanosarcina* is irregular spheroid bodies (1-3 μm in diameter), occuring alone or typically in aggregates of cells (aggregates up to 1000 μm in diameter). Aggregates are small to large spheroid bodies comprising many irregular subunits (Zhilina, 1971, 1976; Zeikus and Bowen, 1975). Optimum growth temperatures are 30-40°C for mesophilic species and 50-55°C for thermophiles.

Acetate, methanol, monomethylamine, dimethylamine, trimethylamine, H_2 / CO_2 and CO are used as growth substrates for methane formation by means of *Methanosarcina* strains. Some strains do not use H_2 / CO_2 as the sole energy substrate.

There exists five mesophilic and only one thermophilic species belonging to the genus *Methanosarcina* namely, *M. barkeri*, *M. acetivorans*, *M. mazeii*, *M. siciliae*, *M. vacuolata* and *M. thermophila*.

The natural order of the living world, however, is readily apparent from the topology of the universal tree: all life falls into one of three primary groupings, formally called domains–the *Bacteria* (or eubacteria), the *Archaea* (formerly archaebacteria), and the *Eucarya* (or eukaryotes) (Woese, 1994). As also seen in Figure 2.3, the *Archaea* is a spesific relative of the *Eucarya* in this new three-domain concept, although the *Eubacteria* and the *Archaea* are both procaryotes.

The *Archaea* domain is divided into three phylogenetically distinct groups depending on the 16S rRNA analysis: *Crenarchaeota*, *Euryarchaeota* and *Korarchaeota*. Although the nucleic acids of the *Korarchaeota* have been detected, there are no organisms isolated or cultured taking place in the phylogenetic tree of *Archaea* (Figure 2.4). The *Archaea* possess three types such as methanogens (prokaryotes that produce methane); extreme halophiles (prokaryotes that live at very high

concentrations of salt); and extreme (hyper) thermophiles (prokaryotes that live at very high temperatures) based on their physiology. The *Crenarchaeota* consists mainly of hyperthermophilic sulfur-dependent prokaryotes and the *Euryarchaeota* contains methanogens and extreme halophiles.

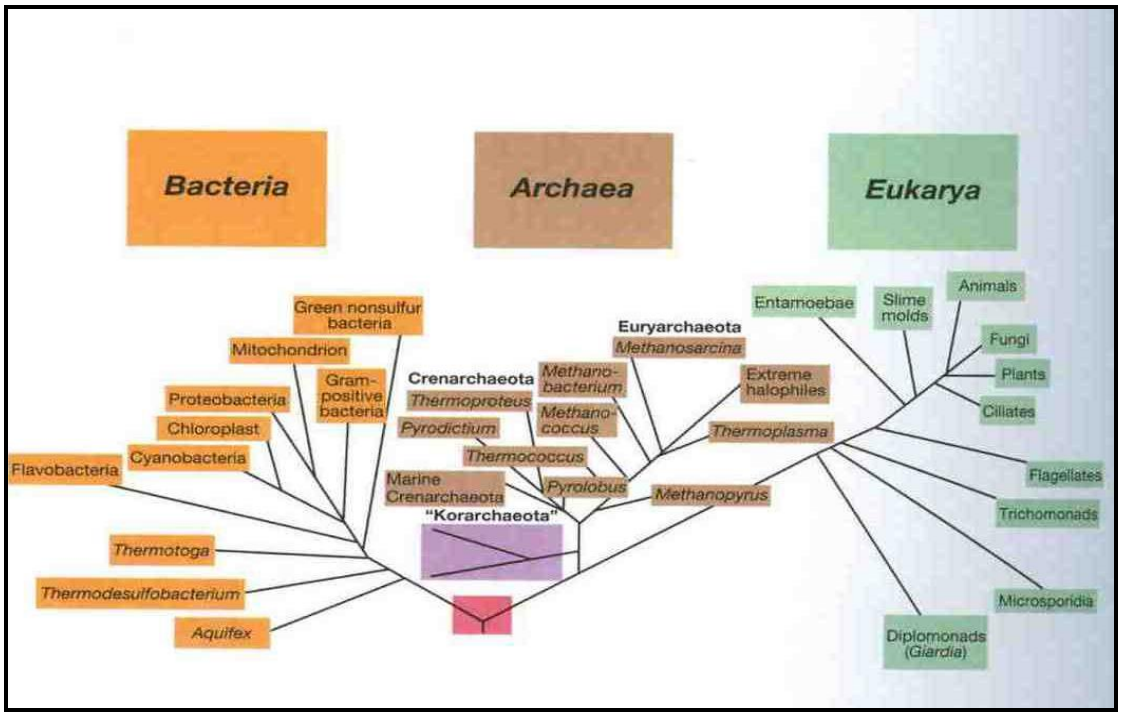


Figure 2.3. Phylogeny of the living world - overview. Universal phylogenetic tree (Madigan *et. al*, 2003).

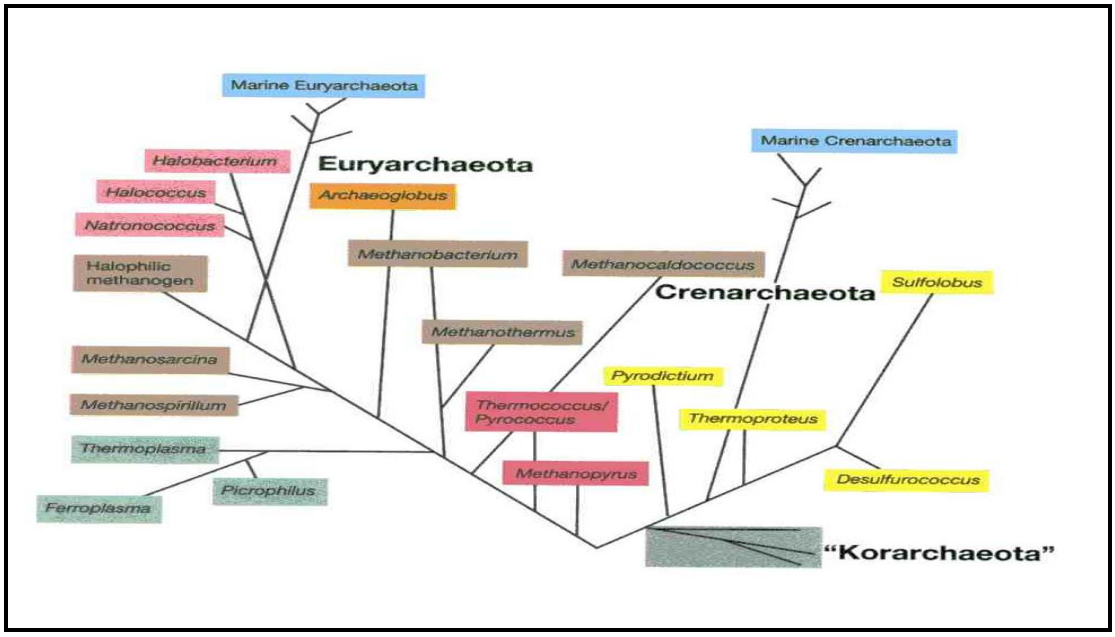


Figure 2.4. Phylogeny of the living world - *Archaea*. Phylogenetic tree of *Archaea* (Madigan *et. al*, 2003).

2.2. Biochemistry of Anaerobic Digestion

The complex or polymeric organic materials such as lipid, cellulose and protein are converted to the immediate substrates of the methanogens (hydrogen, acetate and carbon dioxide). Hydrolytic degradation, fermentation pathways and hydrogen production are carried out by non-methanogenic populations.

The final stage is methanogenesis in anaerobic digestion process. The formation of methane brings two important advantages such as energy gain and low sludge production rate. Carbon dioxide, hydrogen and acetate are degraded by the specialised group of *Archaea* namely, methanogens. The several enzymes and coenzymes which are spesific to methanogens are responsible for this degradation process.

In environments where organic matter is completely mineralised to CH_4 and CO_2, acetate is the major source of methane. Acetate is degraded by aceticlastic reaction, in which the methyl group is cleaved with its hydrogen intact. Members of the two genera of methanogens, *Methanosarcina* and *Methanoseate*, are capable of carrying out this reaction. *Methanosarcina* strains have higher maximum growth rates and may be tolerant of low pH than *Methanoseate* (Maestrojuan and Boone, 1991), but *Methanoseate* has a lower K_m for acetate and consume acetate at a lower concentration than *Methanosarcina* strains (Min and Zinder, 1989; Westermann *et al*., 1989; Fukuzaki *et al*., 1990). When acetate concentrations are very low, *Methanosarcina* strains cannot consume acetate, but rather produce small amounts of it (Westermann *et al*., 1989). The predominance of *Methanosarcina* is possible at low pH values, with rapid dilution rates and higher acetate concentrations. On the other hand, *Methanoseate* is predominant with high pH values or long sludge retention times.

2.3. Environmental Factors in Anaerobic Digestion

In all biological wastewater treatment processes, the effective removal of pollutants and contaminants depends not only on the metabolic potential of the microorganisms but also on the existence of suitable environmental conditions to support these activities (Anderson *et al*., 2003). Environmental factors affecting anaerobic digestion are nutrient composition, temperature, pH, mixing, toxicity and inhibition.

2.3.1. Nutrients

The nutritional demands of anaerobic microorganisms are essential for enzymes and cofactor synthesis catalysing biochemical and metabolic reactions. The nutrients are divided into two different groups as macronutrients (nitrogen, phosphorus, sulphur) and micronutrients (iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt).

2.3.2. Temperature

Temperature is one of the most important environmental factors due to its effect on the cell growth rates and denaturation of the cell structure. There are two distinct temperature ranges associated with anaerobic digestion such as mesophilic and thermophilic (Anderson *et al*., 2003). The optimal mesophilic and thermophilic temperature ranges are 30-37°C and 55-60°C, respectively. Thermophilic reactors have advantages such as high methane production rates, tolerance to high loading rates and low sludge production when compared to mesophilic reactors. On the other hand, thermophilic reactors have also disadvantages over the mesophilic reactors such as less stability than mesophilic reactors, more energy requirements for heating the reactor and high VFA concentration in the effluent.

2.3.3 pH

The maintenance of the pH level within the digester is very important for efficient anaerobic digestion. The optimal pH range is between 6.5 and 7.8. However, there are some exceptions such as anaerobic digestion occuring under pH conditions as low as 3 (Zehnder *et al*., 1982) and as high as 9.7 (Oremland *et al*., 1988).

2.3.4. Mixing

Contact between the organic matter and microorganisms can be improved by an enhanced mixing, leading to higher reactor performance. The level and the type of mixing also affect the growth rate and the distribution of the microorganisms within the sludge, substrate availability and utilisation rates, granule formation and gas production (Anderson *et al*., 2003).

2.3.5. Toxicity and Inhibition

Toxicity has an adverse effect on microbial metabolism, while inhibition is an impairment of microbial function. Many potential subtances could decrease the digestion rate (toxicity) or cause process failure (inhibition). Common toxic substances causing operational failures include heavy metals, alkali and alkaline earth metals, volatile fatty acids, oxygen, ammonia and sulphide (Anderson *et al*., 2003).

2.4. Reactor Configurations

2.4.1. Conventional or completely mixed anaerobic digester

The conventional anaerobic digester is a completely mixed reactor with no solids recycle. This provides that the solids retention time equals to the hydraulic retention time. In these reactors (Figure 2.6), anaerobic bacteria and wastewater are mixed together and allowed to react. The conventional anaerobic digesters are disadvantageous due to the large volume demands and wash-out of the microorganisms in the effluent of the most industrial wastewaters. However, this type of anaerobic digester is appropriate to be used for sludge treatment and for wastewaters containing high solids and organic matter content (Anderson *et al*., 2003).

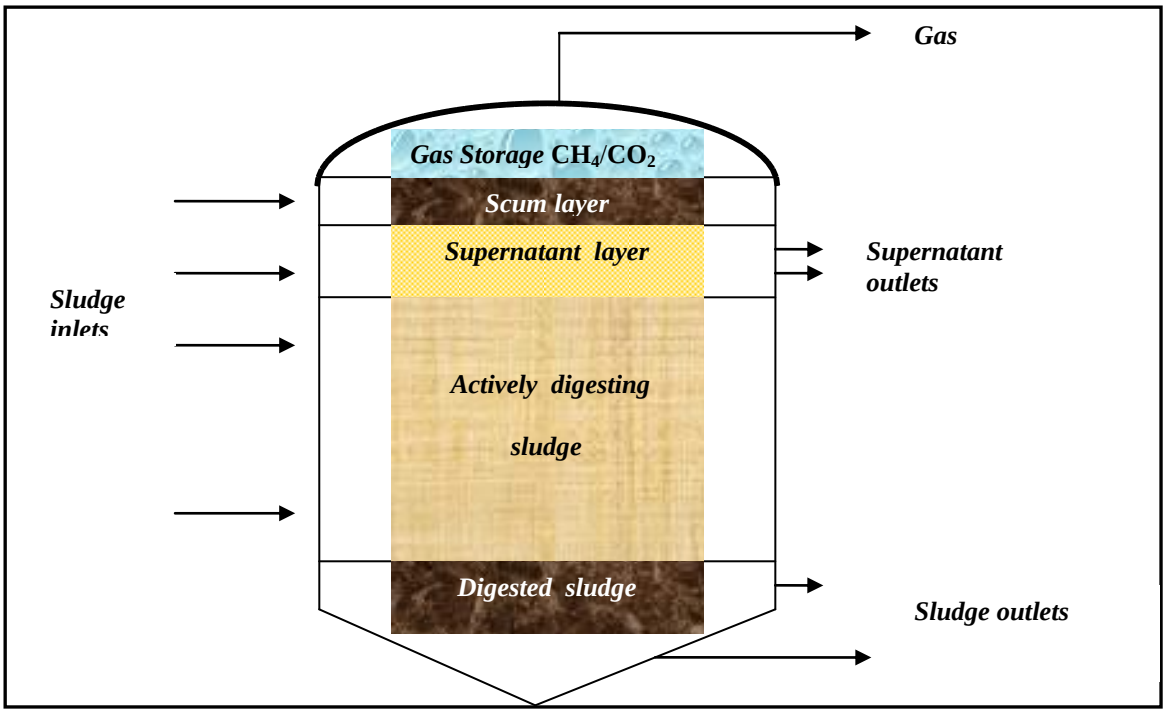


Figure 2.5. Inner workings of conventional single stage anaerobic digestor (Adapted from Metcalf and Eddy, 1991 ; Madigan *et al*., 2003).

2.4.2. Anaerobic contact process

The mechanism of the anaerobic contact process is similar to the activated sludge process. The further contact between microbial sludge and raw waste is provided through settling of sludge and its recycling. The sludge retention time is not equal to hydraulic retention time due to the recycling process. High treatment efficiency is possible with anaerobic contact process. However, some problems might occur related with the settling of anaerobic sludge. For instance, the growth of filamentous bacteria and gas formation by anaerobic bacteria prevent an effective sedimentation of the sudge. But these problems could be overcome with the maintenance of the N/P ratio and with vacuum degasification or thermal shock (Anderson *et al*., 2003).

2.4.3. Anaerobic sequencing batch reactor (ASBR)

It is a batch-fed, batch-decanted, suspended growth system and is operated in a cyclic sequence of four stages; feed, react, settle and decant (Wirtz and Dague, 1996). The limitations of this reactor type are the time consuming settling process and large volume requirement. On the other hand, additional biomass settling stage or solids is not required, further the granule formation is also possible in ASBR after long periods of operation (Anderson *et al*., 2003).

2.4.4. Anaerobic packed bed or anaerobic filter

The anaerobic filter is a fixed-film biological wastewater treatment process. Anaerobic microorganisms attach to a fixed matrix (support medium) and form a biofilm. The wastewater flows upwards through the bed and the dissolved pollutants are absorbed by the biofilm (Young, 1983). The solid seperation and recycle are not required with the use of anaerobic filters. The different types of support material can be used such as plastics, granular activated carbon, sand, granite, quartz and stone. These materials enable high treatment efficiency due to their tolerance to shock loads and operational perturbations. The use of anaerobic filters is suitable for both dilute soluble wastewaters and higher-strength soluble wastewaters that can be diluted by recyling (Wheatley, 1990).

2.4.5. Anaerobic fluidized and expanded bed reactors

The biomass is attached to the surface of small, low spesific gravity particles such as anthracite, high density plastic beads and sand. These particles are kept in suspension

by the upward velocity of the liquid flow (Anderson and Saw, 1986). Effluent is recycled to dilute the incoming waste and to provide sufficient flow-rate to keep particles in suspension. The large surface area of the support particles and high degree of mixing provide a high biomass concentration and efficient substrate uptake kinetics, respectively. However, the limitations of fluidized bed reactors arise from operational difficulties (Wheatley, 1990; van Haandel and Lettinga, 1994).

2.4.6. Upflow anaerobic sludge blanket (UASB) reactor

The biomass in this type of reactor is retained as a blanket or granular matrix, and is kept in suspension by controlling the upflow velocity. The wastewater flows upwards through an expanded bed of active sludge located in the lower part of the reactor, while the upper part contains a three-phase (solid, liquid, gas) seperation system. van Haandel and Lettinga (1994) considered the three-phase seperation device to be the most characteristic feature of the UASB reactor.

2.4.7. Anaerobic baffled reactor (ABR)

This type of reactor combines the advantages of the anaerobic filter and the upflow anaerobic sludge process. Furthermore, this special design is capable of reducing the biomass washout. The ABR can be used for almost all soluble organic wastewater from low to high strength (Polsprasert *et al*., 1992). However, the main disadvantage of this reactor is that it has not been widely used at full-scale.

2.4.8. Two-phase anaerobic digestion

Two-phase anaerobic digestion consists of separate reactors for acidification and methanogenesis. These reactors are connected in series, allowing each phase of the digestion process to be optimised independently since the microorganisms concerned have different nutritional requirements, pyhsiological characteristics, pH optima, growth and nutrient uptake kinetics, and tolerances to environmental stress factors (Cohen, 1983). Two-phase anarobic digestion has some advantages (improvement in process control, disposal of excess fast growing acidogenic bacteria without any loss in slow growing methanogenic bacteria and precise pH control in each reactor, etc.) and disadvantages (high sludge accumulation in the first phase and operational difficulties). The diagrams were demonstrated in Figure 2.7 for all of the anaerobic reactor configurations explained in section 2.4.

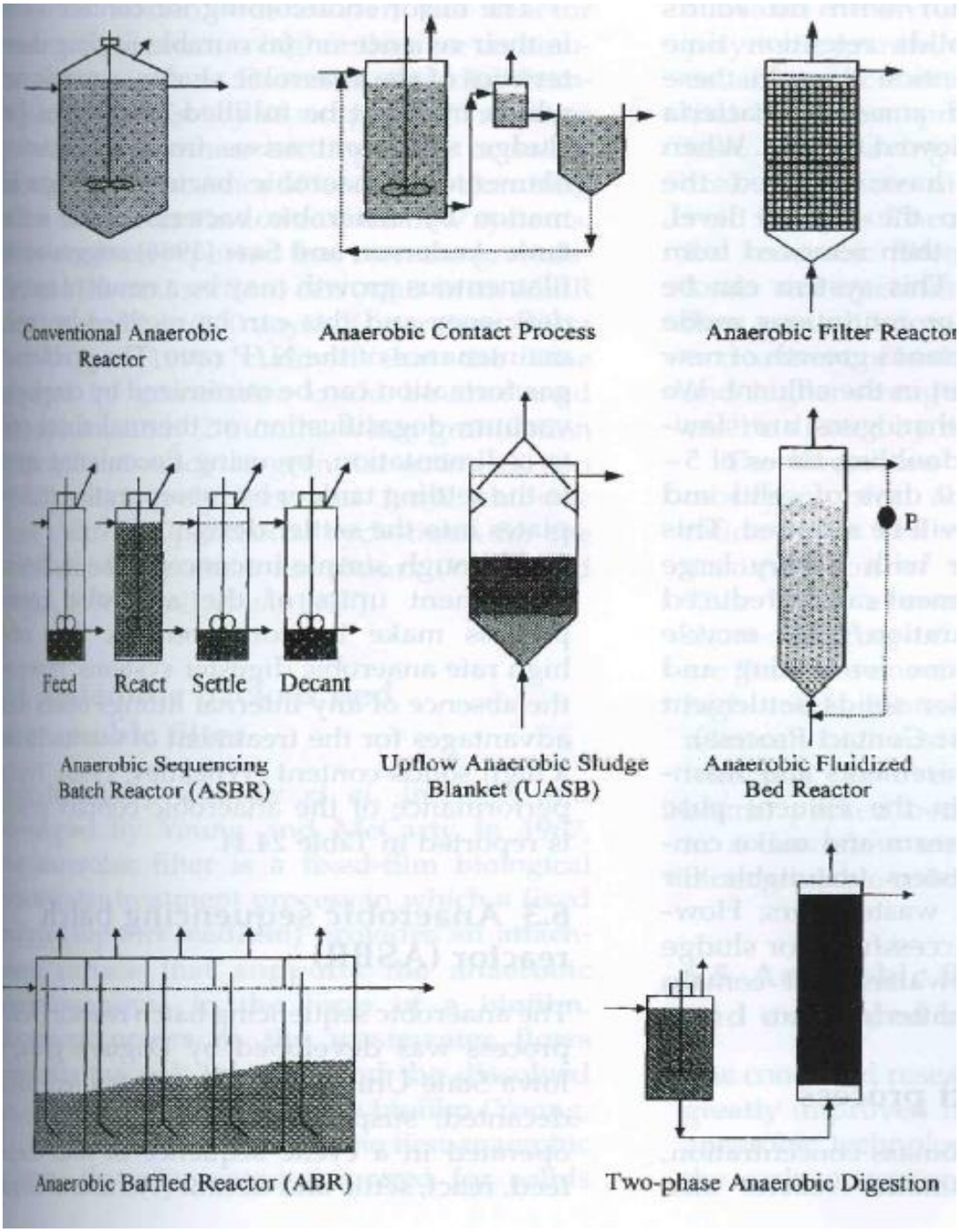


Figure 2.6. Anaerobic reactor configurations used in wastewater treatment (Anderson *et al*., 2003).

2.5. The Methods of Microbial Identification and Quantication

2.5.1. Culture-dependent techniques

Selective growth media is used in classical sludge characterization methods. The Most Probable Number (MPN) method is a technique in which serial sludge dilutions are inoculated in selective liquid media or on solidified agar-media. This method can give very useful information on the number of microorganisms that are able to grow

on artificial media. However, the MPN method is not very accurate for microbial count, if the microorganisms are attached to solid substrates or are associated to each other like threaded bacteria such as the acetoclastic *Methanosaeta* sp. (Grotenhuis *et al.*, 1991; Whitman *et al.*, 1992).

The other method of sludge characterization is direct microscopic analysis. Microbial identification through microsccope techniques are generally based on morphological properties of microorganisms. However, the most bacteria possess distinctive features. An exception are methanogens, they can be identified with epifluorescence microscopy by detecting the factor F420-dependent autofluorescence (Doddema and Vogels, 1978). However, some methanogens, such as *Methanosaeta* do not exhibit autofluorescence (Dolfing *et al*., 1985).

Light, fluorescence, and electron microscopy techniques such as scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM) were used for the examination of microbial biofilms by Surman *et al.* (1996).

All techniques have their special advantages and disadvantages, and the most accurate picture of the true sludge composition can be obtained by 'combined microscopic approach'. For instance, Zellner *et al*. (1993) combined SEM, phase contrast microscopy and epifluorescence microscopy of methanogens in order to study biofilm formation in anaerobic fixed bed reactors.

2.5.2. Culture-independent molecular techniques

Biomarkers, specific antibodies and nucleic acid probes are used for the direct identification of microorganisms in the sludge. For instance, the characterisation of methanogens could be achieved by means of their biomarker such as phospholipid derived ether lipids (PLEL). However, the use of biomarkers is not possible for the microorganisms that lack specific lipid biomarkers (Ringelberg *et al*. 1994). Furthermore, physiologically different microorganisms may possess the same 'specific' lipid biomarker, this can lead to large characterization mistakes in complex microbial ecosystems.

The other microbial identification technique is related with bacterial surface cell wall polymers such as proteins and lipopolysaccharides. These polymers can raise antibodies due to their antigenic properties. If antibodies are labelled with a fluorescent dye or gold particles, in combination with respectively fluorescence or

electron microscopy, they can be used for the specific detection of bacteria (Harlow and Lane, 1988). On the other hand, there exist a limitation such as difficulties in antibody-antigen reaction for this immunodetection technique.

The estimation of prokaryotic diversity in natural habitats was initiated by the application of molecular methods, most of which allowed the recognition of uncultured organisms based on the use of 16S rRNA sequences (Bull *et al*., 2000). Therefore, it might be said that ribosomal RNA-based detection and identification methods have become most important in the unravelling of the microbial composition of anaerobic sludge.

Sequence analysis of the ribosomal RNA or the rRNA-gene has revealed that 16S and 23S rRNA can be used as evolutionary biomarkers (Böttger 1996, DeLong *et al* 1989; Woese 1987). The 16S and 23S rRNA contains both highly conserved as well as highly variable regions. Several rRNA based methods have been developed to identify and quantify microorganisms in complex environments. These methods are i) hybridization with rRNA-based oligonucleotide probes, ii) PCR amplification of rRNA-genes, iii) temperature gradient gel electrophoresis (TGGE) and iv) denaturing gradient gel electrophoresis (DGGE), as also shown in figure 4.1.

Besides, several methods have been developed in order to quantify microorganisms with PCR amplification (Ferré *et al*. 1994) such as i) the use of an internal standard in the amplification of the target DNA (competitive PCR), ii) labelling one of the PCR primers with a reporter molecule (Q-PCR System) and iii) Most Probable Number PCR (MPN-PCR).

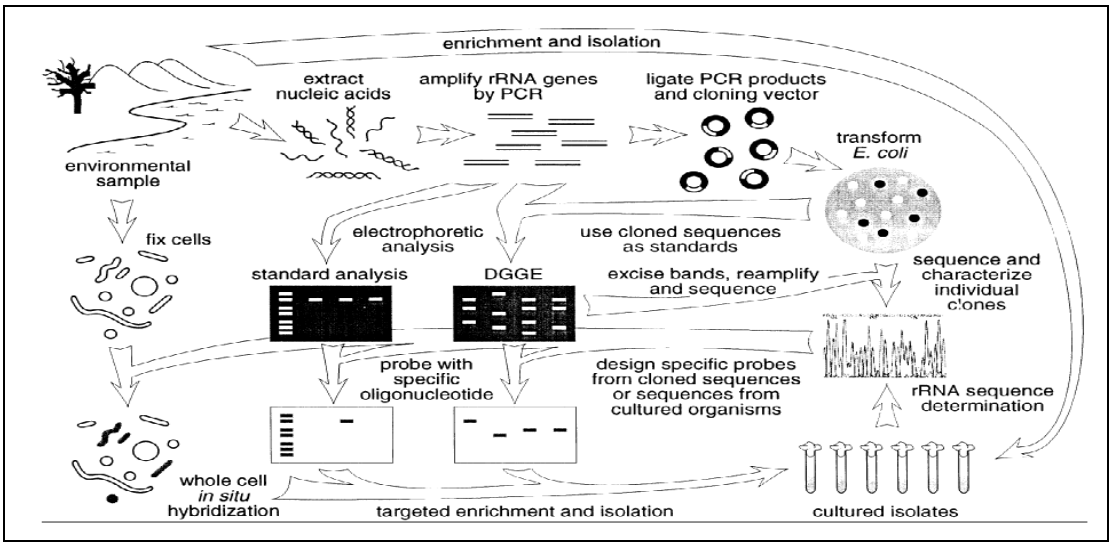


Figure 2.7. Commonly used approaches in molecular microbial ecology (Head *et al*., 1998).

2.6. Fluorescent *in situ* Hybridisation Technique

Fluorescence in situ hybridization (FISH) with rRNA-targeted probes allows phylogenetic identification of bacteria in mixed assemblages without prior cultivation by means of epifluorescence and confocal laser scanning microscopy, or by flow cytometry (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Amann *et al.*, 1990a; Amann *et al.*,1990b; Amann *et al.*, 1996). Oligonucleotide probes are short single stranded oligomers of 15 to 40 nucleotides, which can be synthesized chemically. The oligonucleotide probes are complementary to either variable or conserved parts of the rRNA. They might be either radioactive by ^{32}P-labelling, or chemically linked to fluorescent dyes in order to detect and quantify the microorganisms.

An advantage of fluorescently labelled rRNA probes is that they can be used for in situ hybridization studies (the so called Fluorescent In situ Hybridisation [FISH]-technique), thus making it possible to study the spacial organisation of the microorganisms in the sludge (Elferink *et al*., 1998). However, FISH has also some limitations due to the inactive cells including insufficient ribosome, permeabilisation difficulties and irregular shapes of microorganisms.

The combination of FISH technique and confocal scanning microscopy gives more accurate results about detection and enumeration of microorganisms. This is due to the fact that the whole depth (a merged stack of z-scan slices) of a sample within a given area could be counted by using CLSM. Accordingly, the number of microorganisms per unit area is counted and subsequently converted into volumetric units (Davenport and Curtis, 2004).

Daims *et al.* (1999) devised a sophisticated semi-automated procedure for determining absolute cell numbers in environmental samples to circumvent some of the problems. However, with the careful use of appropriate controls, a CLSM and the appropriate analyses, statistically valid cell counting may be achieved using less complicated methods (Davenport *et al*., 2000).

CHAPTER 3. MATERIALS AND METHODS

3.1. Sampling

Anaerobic sludge samples were taken both from a full-scale conventional single-stage anaerobic digester and a laboratory-scale anaerobic membrane bioreactor seeded with the digested sludge of the same full-scale anaerobic digester. After collection, both of the samples were immediately fixed with absolute ethanol (1:1, vol/vol) in universal bottles (20ml) and transported to the laboratory in an ice box and kept at $-20°C$ until PFA fixation.

3.1.1. Full-scale

The sample was collected from a conventional single-stage anaerobic digester at Hexham Sewage Treatment Works, Hexham, United Kingdom. There exists one mesophilic anaerobic digester possessing 1000m^3 of volume at Hexham STW. Sludge is recirculated to a heat exchanger, which provides a further mixing to supplement the unconfined gas mixing. Digested sludge is held in consolidation tanks prior to land application. The pH values of raw sludge and digested sludge are 5.6 and 6.9, respectively. Volatile fatty acid (VFA) concentration is 40 mg/ℓ on the February 11, 2005 which is the sample collection date (Personal communication with Ivan Jepson who is the Environmental Information Co-ordinator of Northumbrian Water). the anaerobic digester , the digested sampling point and the schematic flow diagram of Hexham digestion plant were shown in Figure 3.1.

3.1.2. Lab-scale

The sludge sample was collected from a lab-scale anaerobic membrane bioreactor treating brewery wastewater seeded with mesophilic sewage sludge of the full-scale conventional anaerobic digester. This experimental system, shown in Figure 3.2 has been operated in the mesophilic temperature range. The system is capable of achieving 98% chemical oxygen demand (COD) removal and 98% total organic carbon (TOC) removal due to the combination of anaerobic bioreactor with membrane unit, at an imposed organic loading rate (OLR) of 1.0-1.5 kg.COD/ℓ.day. Hydraulic retention time (HRT) is 3 days.

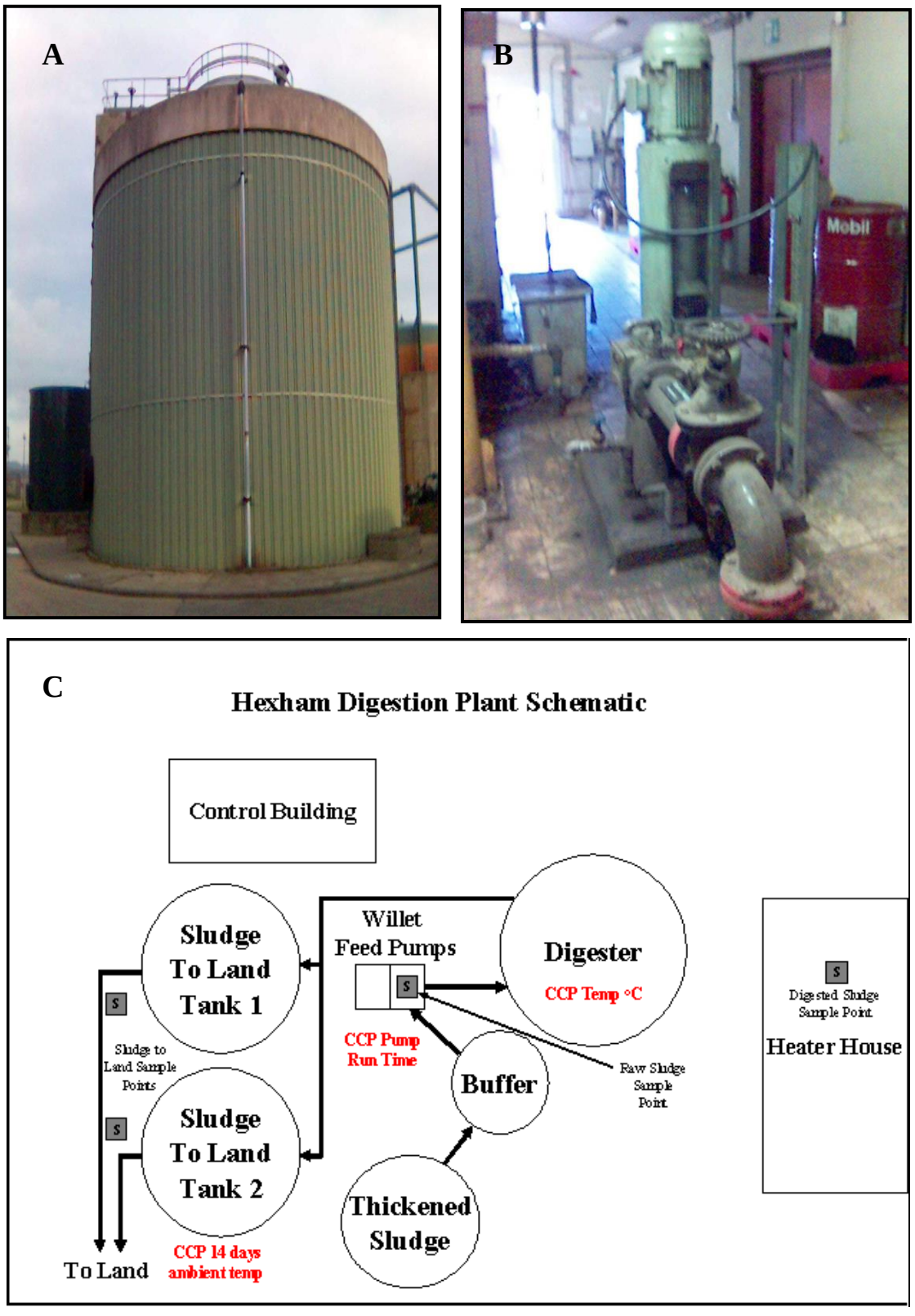


Figure 3.1.A. Conventional single stage anaerobic digester at Hexham Sewage Treatment Works (Hexham, United Kingdom). B. The digested sludge sample point of this anaerobic digester. C. Schematic flow diagram of Hexham digestion plant. (CCP refers to Critical Control Point within the sludge process, such as temperature or retention time).

The amounts of CH_4 and CO_2 produced after influent COD removal were 9.1 ℓ and 1.9 ℓ, respectively. The pH values measured on sampling day for anaerobic bioreactor and permeate/effluent tank were 6.84 and 6.9, respectively. Acetic acid and propionic acid concentrations were 8.75 mg/ℓ and 1.73 mg/ℓ for anaerobic bioreactor, further acetic acid and propionic acid concentrations of permeate/effluent tank were 5.85 mg/ℓ and 1.35 mg/ℓ, respectively.

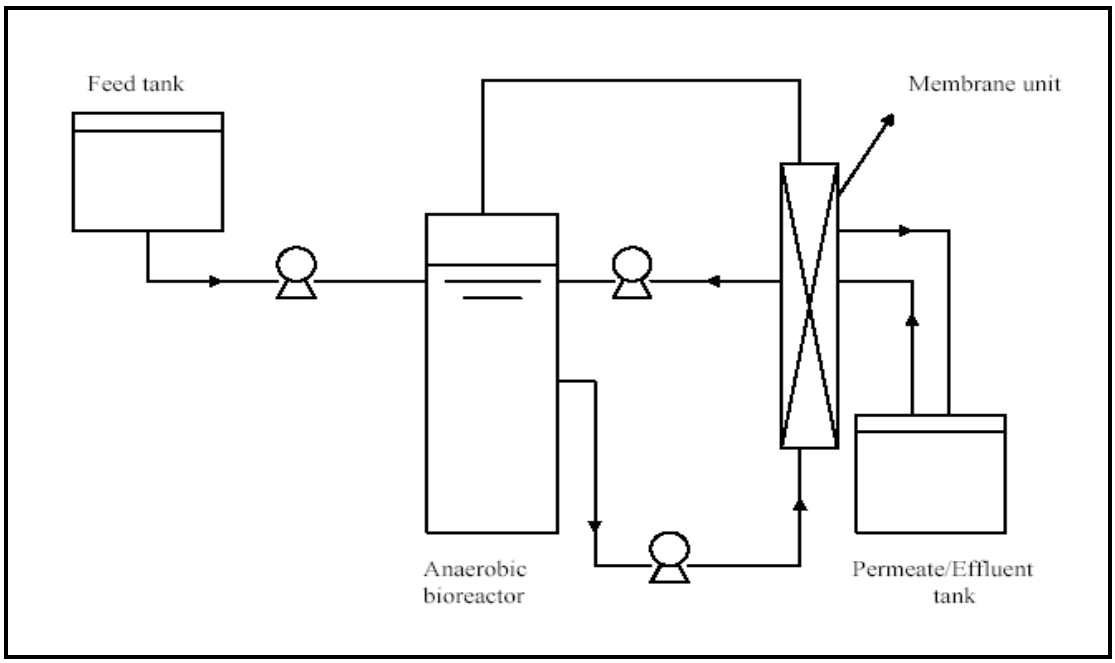


Figure 3.2. Schematic diagram of the lab-scale anaerobic membrane bioreactor treating brewery wastewater (with permission of A. Yuzir).

3.2. Preparation of Solutions

The solutions presented in Table 3.1 were prepared to use for different steps of FISH technique including fixation, hybridisation and washing in this study.

3.3. Total Microbial Counts with Fluorescent DAPI Staining

Total microbial counts were determined with DAPI which stains nucleic acids in order to estimate the appropriate cell concentration in an environmental sample for hybridisation procedure. DAPI gives the whole amount of microbial cells due to its capability of staining all microorganisms present in an environmental sample. DAPI (Sigma-Aldrich Company Ltd., Poole, UK) solution was prepared at 3.3 μg ml^{-1} concentration and was stored at 4°C in the dark.

Table 3.1. The solutions used in fixation, hybridisation and washing steps of FISH.

	In 100 ml distilled water	**In 100 ml sterilised water**	**pH**	**Autoclave**
10 x PBS (phosphate buffer saline)	7.6 g of 1.3 M NaCl, 1.36 g 100mM $KH_2 PO_4$	-	7.4	+
200 mM Tris-HCl	2.42 g of Tris	-	7.2	+
4.5 M NaCl	26.3 g of NaCl	-	-	+
10% SDS (sodium dodecyl sulphate)	-	10 g of SDS	7.2	-
0.5 M EDTA (disodium ethylenediaminotetra acetate.2H_2O)	18.61 g of EDTA	-	8.1	+

Three dilution rates of each sample were prepared with MilliQ (double sterilised water) and DAPI. The diluted solutions were mixed and incubated for 20 minutes at room temperature in the dark. 30 µl of each solution stained with DAPI was filtered with addition of 70 µl of MilliQ water through black polycarbonate filters (Millipore, USA) having 0.2 µm pore size on the sterile millipore stainless steel filter unit by starting with the most diluted solution. After filtration of the solution , the filter was removed and placed over a small drop of Citifluor (Citifluor Ltd, Canterbury, UK) laying on a clean slide. Citifluor is an antifadent which prevents photo-bleaching of fluorescence DAPI stain. The surface of filter was allowed to air dry for a few minutes to hinder the sample movement under the epifluorescence microscope. Another small drop of Citifluor antifadent was added prior to placing a cover-slip over the preparation. The edges of the cover-slip was sealed using nail varnish to avoid the surface tension of the oil due to the ×100 oil immersion lens of epifluorescence microscope during epifluorescence microscopy. This sealing process was carefully done without smudging the nail varnish to the samples. The reason of this care is to protect the samples from its autofluorescence characteristics.

After slide preparation, the epifluorescence microscopy was performed on the samples by using Olympus BX40 microscope (Olympus Optical Co., Ltd., Tokyo,

Japan). Cells taking part within graticule grid limits were counted for 20 different fields of view (FOV). The total number of the cells per ml was calculated with the formula according to the method of Kepner and Pratt (1994) given in equation 3.3.1:

$$\text{Total number of cells per ml} = \frac{\text{Mean number of cells per FOV} \times \text{Total area of filter } (132.73\ mm^2)}{\text{Area of FOV } (0.01\ mm^2) \times \text{Volume of sample applied } (0.03\ ml) \times \text{Dilution used}} \quad (3.3.1)$$

Finally, the images of DAPI stained samples were captured by means of Olympus CW - 95 digital camera mounted onto the epifluorescence microscope.

3.4. Permeabilisation and fixation

Standard paraformaldehyde (PFA) fixation was used for permeabilisation and fixation of sewage sludge samples taken from a lab-scale and a full-scale anaerobic digestors in this study.

3.4.1. Paraformaldehyde fixative solution preparation

10 × PBS , 10M NaOH and PFA were added into the sterile distilled water, after heating up to 60°C. Dissolved paraformaldeyde solution was cooled on ice. pH of the solution was adjusted to 7.2 and the solution was filtered through a filter 0.2 μm pore size.

3.4.2. Fixation

1 ml of the sample (sludge + absolute ethanol) (1:1, v/v) was washed with PBS and resuspended in 0.25 ml of PBS. 3 volumes of PFA (4%, wt/vol) was used for each volume of cell suspension. Accordingly, 0.75 ml of PFA fixative solution was added into suspension. The preparation was incubated overnight at 4°C. After fixation, cells were washed with PBS and 1 ml of PBS and absolute ethanol (1/1, v/v) was added. This suspension was stored at −20°C until the hybridisation process.

3.5. Hybridisation

3.5.1. Fluorescently labelled 16S rRNA targeted oligonucleotide probes

The mixture of EUB338I, EUB338II and EUB338III probes were used in order to detect all *Bacteria* in sludge samples. *Archaea* domain-spesific and two different genera of methanogenic *Archaea* namely *Methanosarcina* and *Methanoseate*

(responsible for acetoclactic reaction) spesific probes were also used to detect and enumerate the abundance of the total *Archaea* and particularly acetoclastic methanogens. All of the probes were obtained from Thermo Electron Corporation (Germany) in this study. The properties of the probes were summarised in Table 3.3.

3.5.2. Fluorophores of oligonucleotide probes

The sulfoindocyanine dyes, indocarbocyanine (Cy3) and indodicarbocyanine (Cy5) were used to label the *Archaea* domain- and two different methanogenic archaeal genera-spesific probes, respectively. Since, these fluorophores possess high photostability and narrow emmission bands. Besides, one of the fluorescein-derivates fluorescein isothiocyanate (FITC) was also used for labelling of the *Bacteria* domain-spesific probes. Table 3.2 demonstrates the wavelengths and laser types according to the fluorophores for image acquisition.

Table 3.2. The wavelengths and laser types for each fluorophore during image acquisition processes for *in situ* detection of microorganisms in this study.

Fluorophore or labelling reagent	**Colour of fluorophore**	**Excitation wavelength (nm)**	**Emmission wavelength (nm)**	**Type of laser**
FITC	Green	488	499-550	Ar
Cy3	Red	543	555-631	He/Ne
Cy5	Blue	633	650-750	He/Ne

Table 3.3. Fluorescently labelled 16S rRNA targeted oligonucleotide probes used in this study.

Probe	**Target organisms (rRNA target, position)**	**Characteristic substrates**	**Sequence (5'-3')**	**Labelling agent**	**Reference**
EUB338I	Most *Bacteria* (16S, 338-355*)		GCTGCCTCCC GTAGGAGT	FITC	Amann *et al*., 1990
EUB338II	*Planctomycetales* and other *Bacteria* not detected by EUB338 (16S, 338-355*)		GCAGCCACC CGTAGGTGT	FITC	Daims *et al*., 1999
EUB338III	*Verrucomicrobiales* and other *Bacteria* not detected by EUB338 (16S, 338*-355*)		GCTGCCACCC GTAGGTGT	FITC	Daims *et al*., 1999
ARC915	*Archaea* (16S, 915*-934*)		GTGCTCCCCC GCCAATTCCT	Cy3	Amann *et al*., 1995
MX825	*Methanosaeta* (16S, 825*-847*)	Use only acetate; generally have low minimum threshold, *K*, and μ_{max} values	TCGCACCGTG GCCGACACC TAGC	Cy5	Rocheleau *et al*., 1999
MS821	*Methanosarcina* (16S, 821*-844*)	Use acetate and other substrates (H_2 - CO_2 ,methanol,and methylamines); generally have high minimum threshold, *K*, and μ_{max} values for acetate	CGCCATGCCT GACACCTAG CGAGC	Cy5	Rocheleau *et al*., 1999

*The numbers refer to E.coli numbering (Brosius *et al*., 1981)

3.5.3. Optimisation of hybridisation conditions

As demonstrated in Table 3.4, hybridisation conditions and subsequent wash steps were optimised for probes used in each dual hybridisation in order to obtain the strongest binding.

After PFA fixation and permeabilisation, 200 µl and 100 µl of the fixed cells were taken and serially dehydrated in successive increasing ethanol concentrations (60, 80 and 100%) for three minutes each. Following this, cells were resuspended by adding 36 µl of simple hybridisation buffer (900mM NaCl, 0.1% SDS, 20mM Tris-HCl; pH 7.2, X % deionised formamide). 2 µl of the first probe (50 ng μl^{-1}) and 2 µl of second probe (50 ng μl^{-1}) were added to obtain 40 µl of total volume. The cells were incubated overnight at the optimal hybridisation temperature. After the hybridisation period, the cells were washed twice with 0.5 ml of appropriate wash buffer (X mM NaCl, 5 mM EDTA; pH 8.0, 20 mM Tris-HCl; pH 7.2, 0.1 % SDS) and were incubated for 15 minutes at the optimal washing temperature. The cells were also washed with MilliQ water and finally resuspended in 100 µl of MilliQ water.

Accordingly, three dual hybridisations, negative and anti-bacterial controls were carried out for anaerobic sludge samples taken from the full-scale and lab-scale digesters. The amounts of probes used in each dual hybridisation and antibacterial control were demonstrated in Table 3.5 for the sludge samples.

Table 3.4. Optimum hybridisation conditions and hybridisation wash step for each probe used in this study.

Probe	T_d (°C) (Hybridisation temperature)	% Formamide [v/v] in simple hybridisation buffer	T_d (°C) (Washing temperature)	Concentration [mM] of monovalent cations in wash buffer
EUB338 mix_{FITC} (green)	46	20	48	225
$ARC915_{Cy3}$ (red)	46	30	48	112
$MX825_{Cy5}$ (blue)	46	20	48	225
$MS821_{Cy5}$ (blue)	46	20	48	225

Table 3.5. Dual hybridisations, negative and positive controls conducted for each anaerobic sludge sample in this study.

	I. Probe (µl)	II. Probe (µl)	HB (µl)
Dual Hybridisation I	$ARC915_{Cy3}$ (red) 2	EUB338 mix_{FITC} (green) 2	Simple HB 36
Dual Hybridisation II	$ARC915_{Cy3}$ (red) 2	$MX825_{Cy5}$ (blue) 2	Simple HB 36
Dual Hybridisation III	$ARC915_{Cy3}$ (red) 2	$MS821_{Cy5}$ (blue) 2	Simple HB 36
Negative control	0	0	Simple HB 40
Anti-bacterial control	Anti Eub probe 2	-	Simple HB 38

The dual hybridisation procedure carried out in this study was presented through the basic principles of FISH such as fixation, hybridisation, washing and detection in Figure 3.3.

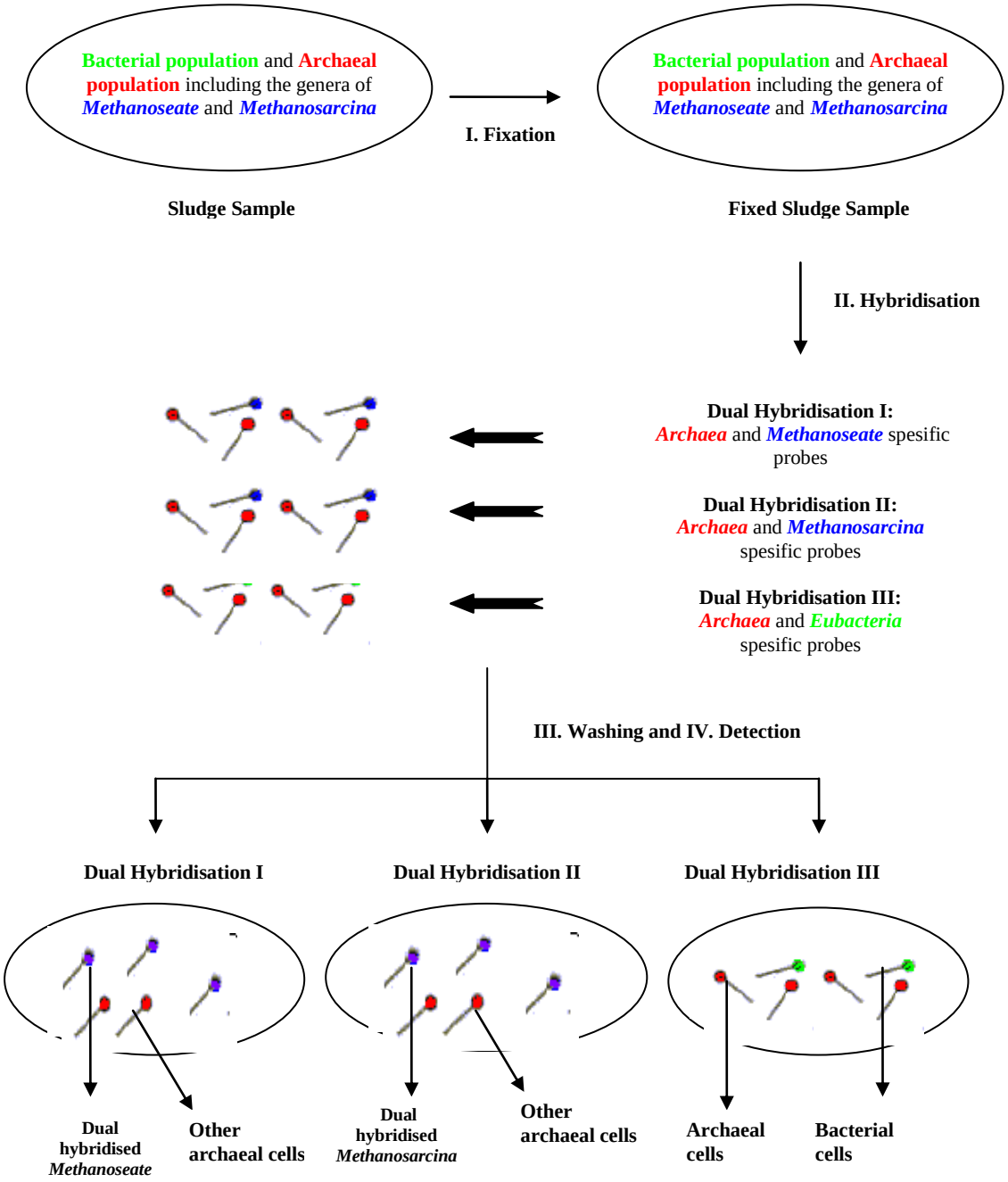


Figure 3.3. Dual hybridisation procedure based on principle steps of fluorescent *in situ* hybridisation used in this study. Each colour (red, blue and green) of dots indicates different fluorescent dye molecules that are linked to different 16S rRNA targeted oligonucleotide probes (*Archaea*, *Bacteria*, *Methanoseate* and *Methanosarcina* spesific probes were used.) (Adapted from Amann and Schleifer, 2003).

3.6. Microscopic observation

3.6.1. Preparation of gelatine-coated slides

A rack of teflon imprinted glass microscope slides including eight wells were cleaned through immersion into 10% KOH (w/v) and 95% ethanol solution. The rack was removed and the solution was replaced with distilled water after one hour. The slides were submerged in the distilled water for 30 seconds and shaken to remove the excess water and they were submerged in the fresh water. This process was repeated further three times. The rack of slides were left to air dry. Gelatine coating solution (0.1% gelatine, 0.01% $CrK(SO_4)_2$ w/v) was prepared in hot distilled water heated up to 70°C in a plastic microscope slide box placed in a water bath while the slides are drying. The rack of dried slides were immersed into the coating solution for three minutes. They were left to air dry for five minutes. This process was repeated further three times. Lastly the rack of slides were left to air dry and stored in the dark at 4°C.

3.6.2. Preparation of slides for microscopic view

10 µl aliquot of the hybridised sample was added to a Teflon imprinted gelatine-coated slide and allowed to dry in the hybridisation oven at 30°C, following a small drop of Citifluor antifadent (Citifluor Ltd, Canterbury, UK) was mounted onto the sample. A coverslip was placed and the edges of coverslip were sealed with nail varnish. The slides prepared were stored in the dark at 4°C until identification with the confocal laser scanning microscopy.

3.6.3. Digital image acquisition

Confocal microscopy was performed using a microscope (CLSM, Leica TCS SP2 UV, Leica microsystems, Heidelberg, Germany) equipped with oil immersion type of objective lens ×63 and with numerical aperture (NA) setting of 1.32. Two-dimensional all-in-focus projections were obtained through Leica TCS SP2 UV CLSM in combination with Leica Confocal Software (Version 2.5). Figure 3.4 demonstrates confocal scanning microscope used in this study. Single optical sections were recorded by using a 633 nm HeNe laser for Cy5, a 543 nm HeNe for Cy3 and a 488 nm Ar ion laser for FITC to optimize the settings of pinhole size, contrast and brightness for each fluorescence channel independently. Optical section thickness was determined as 1.25 µm for this study. Sequential scanning was performed in order to avoid cross-talk between channel emmissions for dual-labelled specimens. A

microscopic field was randomly selected. Following this, z sectioning was started by using optimised fluorescence channels. The number of z sections were determined between 7 - 18 sections according to the sample thickness. The series of optical sections were visualised as a gallery. The two-dimensional (all-in-focus) projection was obtained through series of optical sections and saved as a TIFF file for each emmission channel seperately. Furthermore, TIFF files were loaded in two channels in order to obtain superimposed all-in-focus images for each dual hybridised sample (Wagner *et al*., 1998).

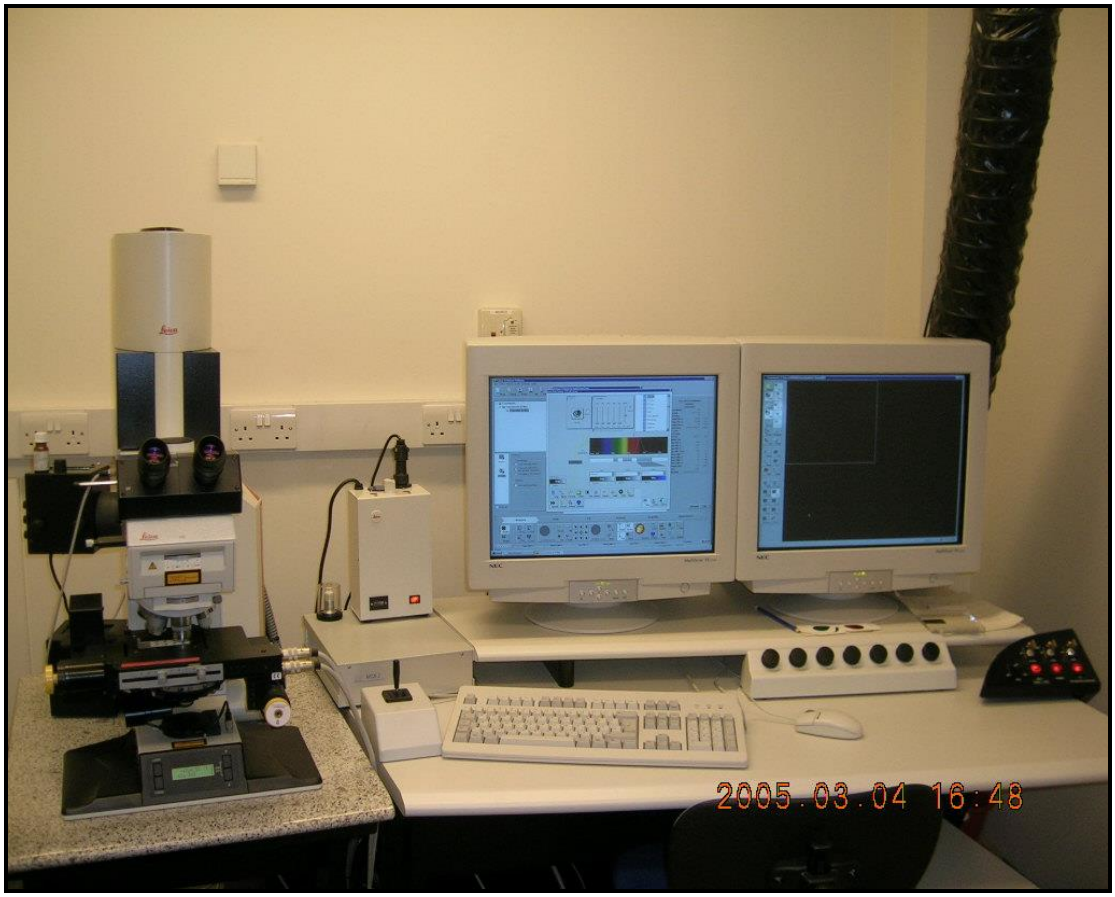


Figure 3.4. Leica TCS SP2 UV confocal laser scanning microscope used in this study. (Photograph taken with permission of Dr. Trevor A. Booth)

3.7. Quantitative FISH procedure

After sampling and hybridisation, the specific cells in the hybridised sample spot were viewed using CLSM. Five different fields of view (FOV) were recorded and counted, as also shown in Figure 3.5.

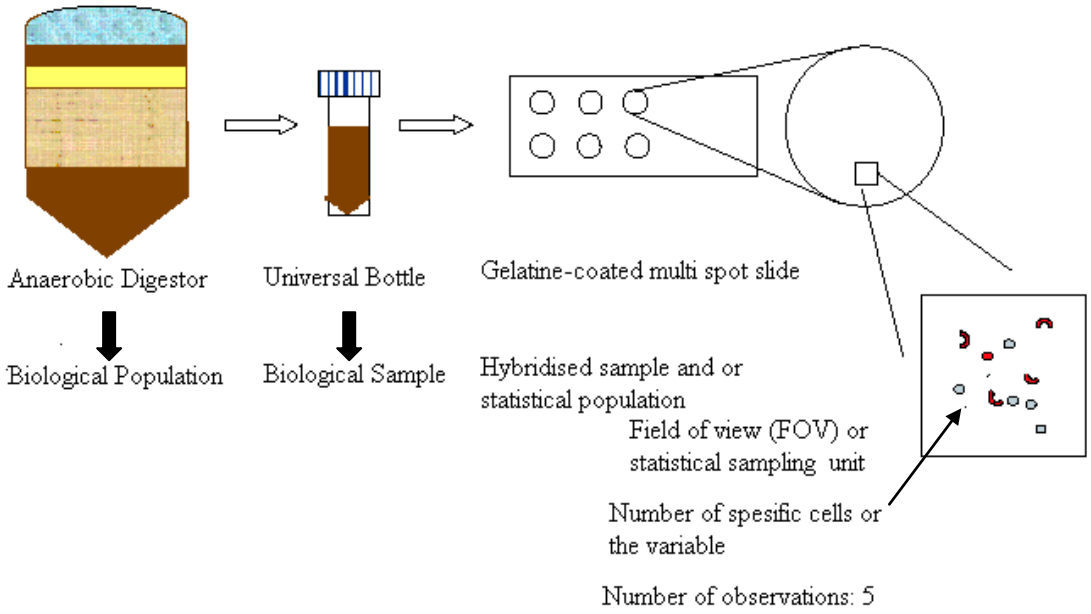


Figure 3.5. A schematic diagram showing the analytical process of a sample using quantitative FISH, from collection of the biological population, through FISH, to quantification of specific cell counts for statistical analysis. The terminology of each step is highlighted. In quantitative FISH the statistical population is usually deemed to be a good estimator of the biological population (Adapted from Davenport and Curtis, 2004).

The numbers of *Archaea* , *Methanoseate, Methanosarcina* and *Bacteria* cells per milliliter was calculated by means of the formula given in equation 3.3.2:

$$\text{Total number of cell per ml} = \frac{\text{Number of cells per FOV} \times \text{Area of sample spot } (19.63\ mm^2)}{\text{Area of FOV } (mm^2) \times \text{Volume of sample applied } (10\ \mu l) \times \text{Dilution used}} \quad (3.3.2)$$

3.8. Statistical analysis

The scheme depicted in Figure 3.6 gives the process for the data analysis of count data (*Archaea*, *Methanoseate*, *Methanosarcina* and *Eubacteria* cell counts) following quantitative FISH technique on the samples. This schematic process is followed to statistically evaluate and compare two or more means of a population counted using quantitative FISH. The count data must be made from randomly chosen field of view (Davenport and Curtis, 2004).

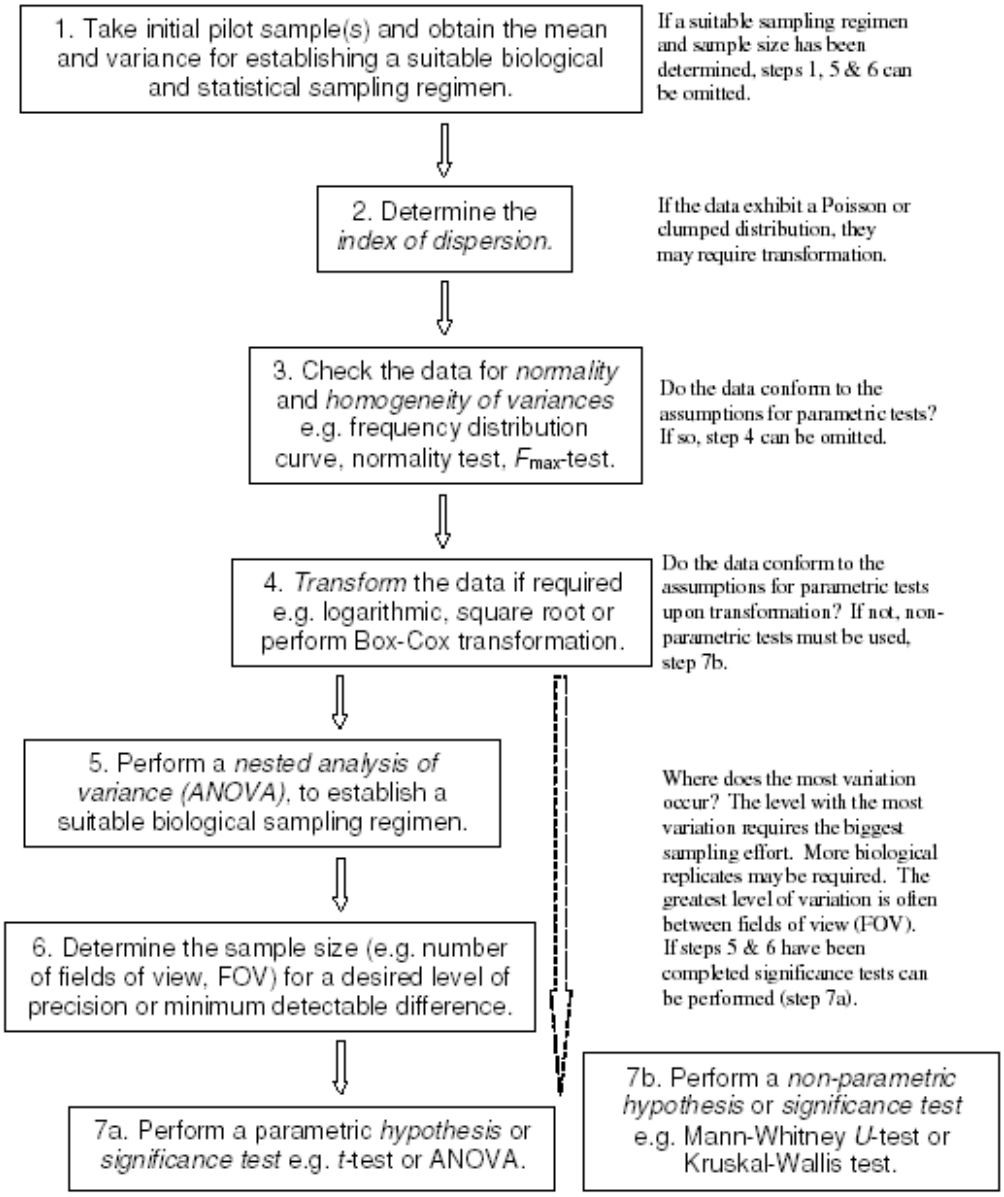


Figure 3.6. A schematic flow diagram showing the steps involved for the statistical analysis of count data from quantitative FISH (Davenport and Curtis, 2004).

CHAPTER 4. RESULTS

4.1. Fluorescent Staining Using DAPI

Samples were stained with fluorescence DAPI nucleic acid stain to determine total cells present in the sludges of the full-scale and the lab-scale anerobic digesters. DAPI stained cells were observed and enumerated under epifluorescence microscope, as also illustrated in Figure 4.1 and 4.2. The concentrations of the whole cell present in the full-scale and the lab-scale anaerobic digesters were calculated as $3.19 \pm 0.5 \times 10^9$ cells ml^{-1} and $4.03 \pm 0.53 \times 10^9$ cells ml^{-1}, respectively.

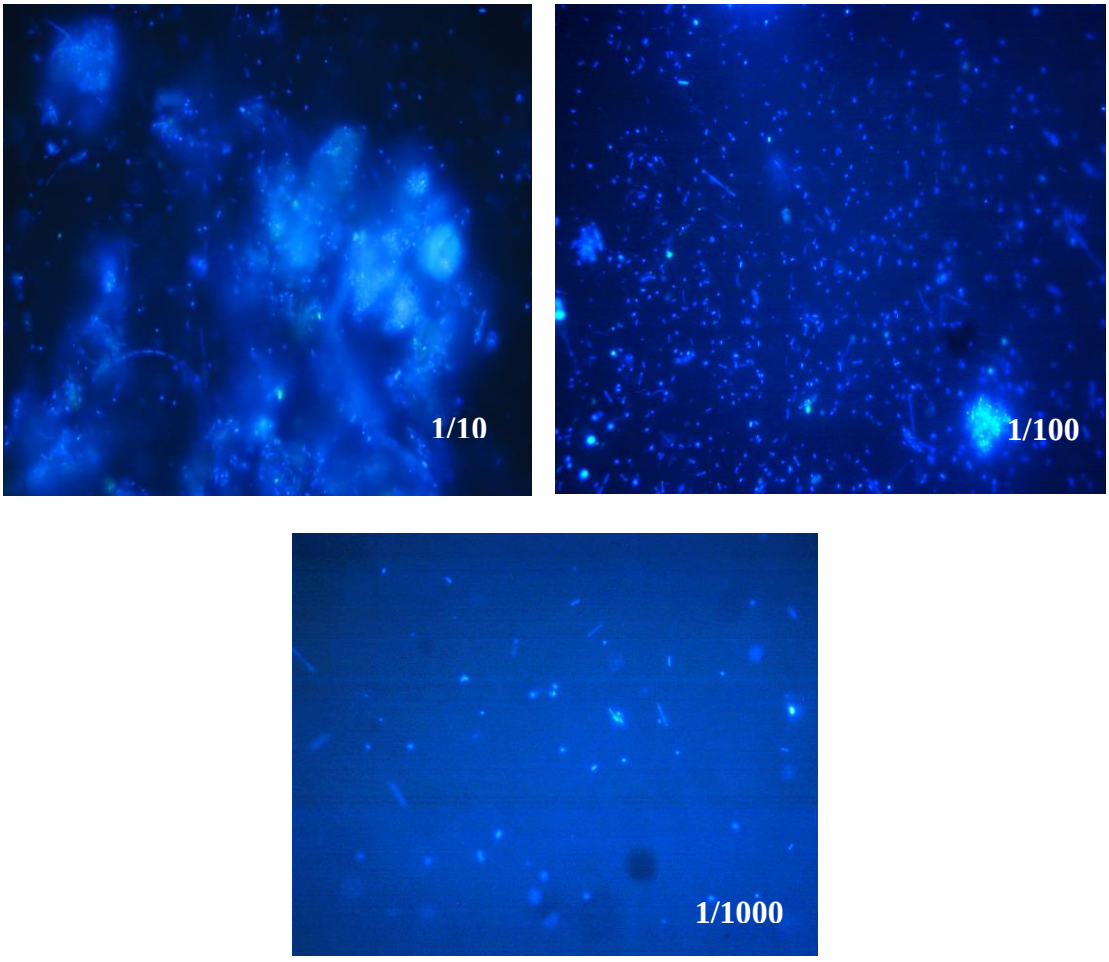


Figure 4.1. Images of DAPI stained cells at three different dilution rates (1/10, 1/100 and 1/1000) for the sewage sludge sample taken from the full-scale mesophilic anaerobic digester at Hexham Sewage Treatment Works.

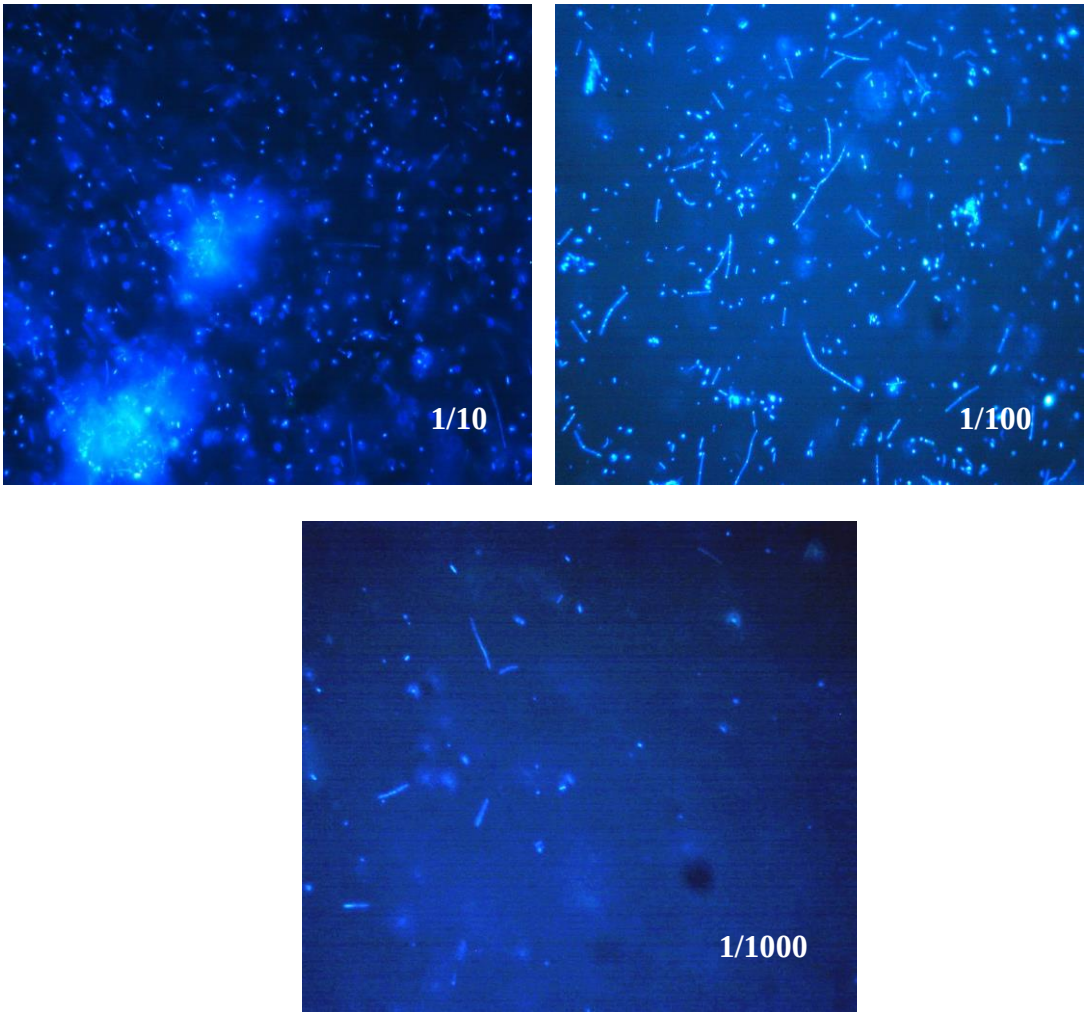


Figure 4.2. Images of DAPI stained cells at three different dilution rates (1/10, 1/100 and 1/1000) for the sewage sludge sample taken from the lab-scale anaerobic membrane bioreactor seeded with Hexham sludge.

4.2. Dual Hybridisations with Flourescently Labelled Oligonucleotide Probes

Three dual hybridisations were carried out for each anaerobic sewage sludge sample. The results of dual hybridisation I using $Arc915_{Cy3}$ (red) and $Eub338_{FITC}$ mix (green), dual hybridisation II using $Arc915_{Cy3}$ (red) and $MX825_{Cy5}$ (blue) and dual hybridisation III using $Arc915_{Cy3}$ (red) and $MS821_{Cy5}$ (blue) are shown in Figures 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8 for the anaerobic sludge samples of the full-scale and the lab-scale digesters.

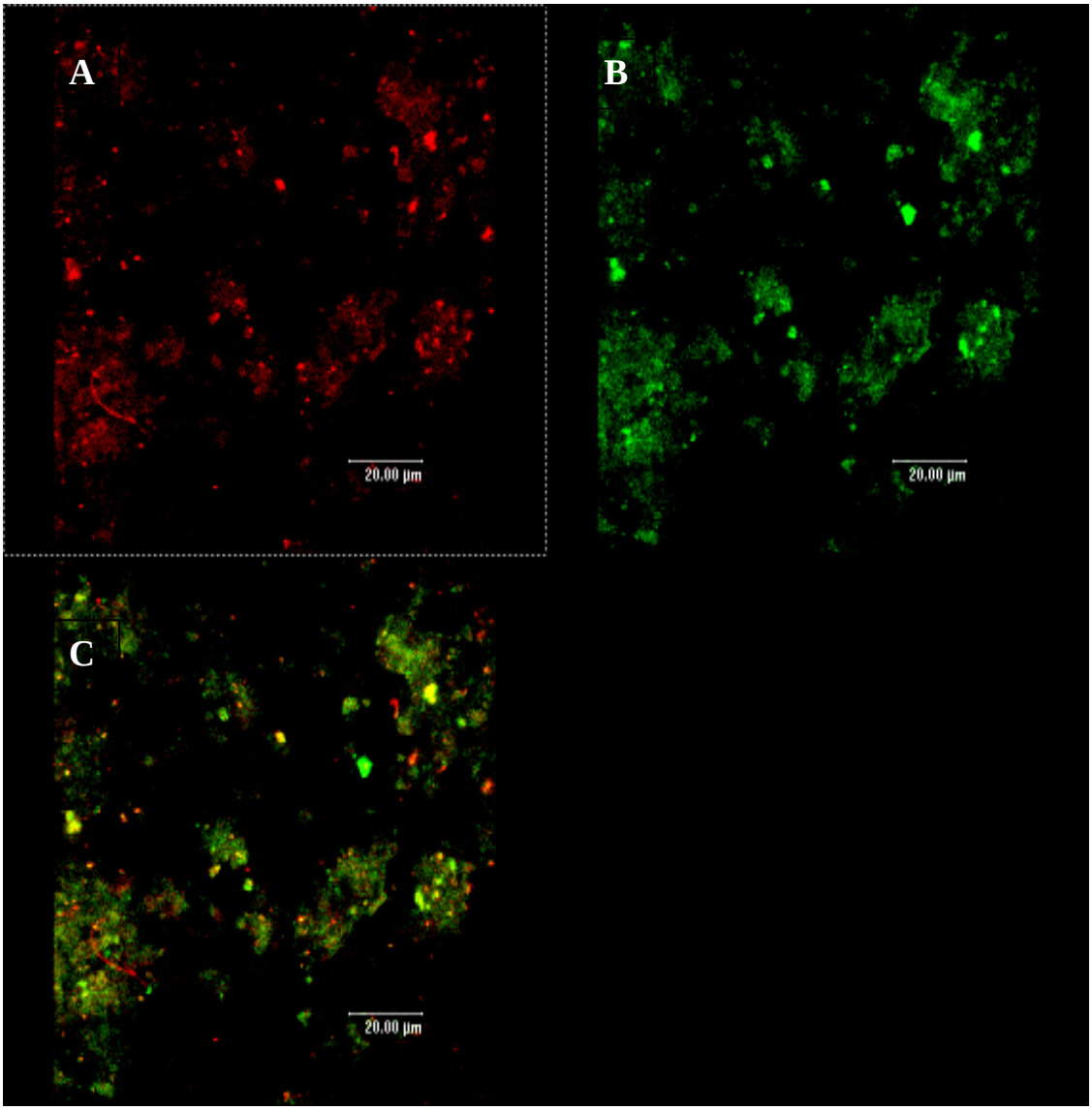


Figure 4.3. CLSM image of full-scale sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $Eub338_{FITC}$ mix (green) probes (Scale bar, 20 µm).

As also seen in Figure 4.3, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* and *Bacteria* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of *Archaea* and *Bacteria* in one image (C). Sample was taken from mesophilic anaerobic digester at Hexham Sewage Treatment Works.

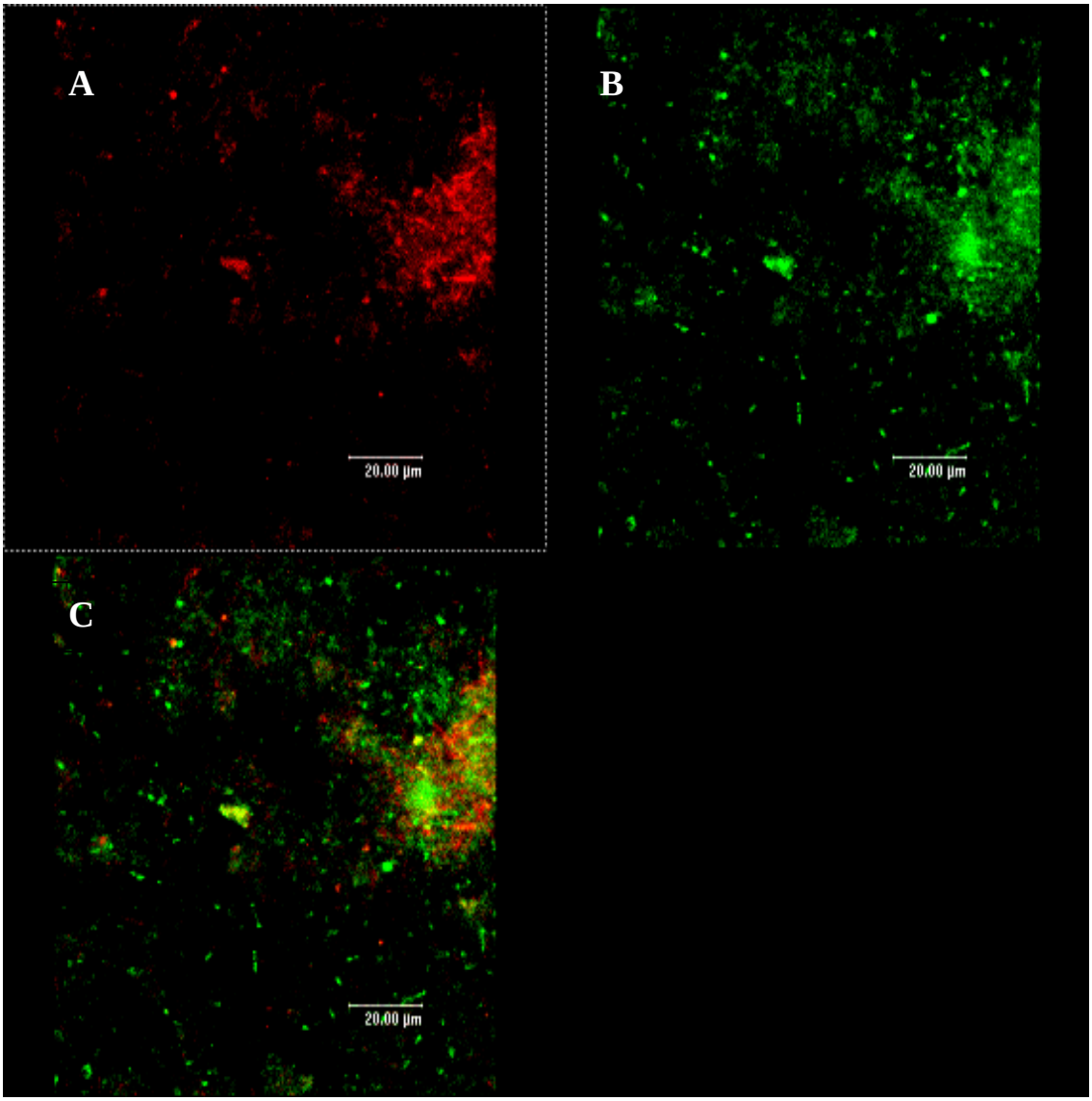


Figure 4.4. CLSM image of lab-scale sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $Eub338_{FITC}$ mix (green) probes (Scale bar, 20 µm).

As also seen in Figure 4.4, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* and *Bacteria* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of *Archaea* and *Bacteria* in one image (C). Sample was taken from lab-scale anaerobic membrane bioreactor.

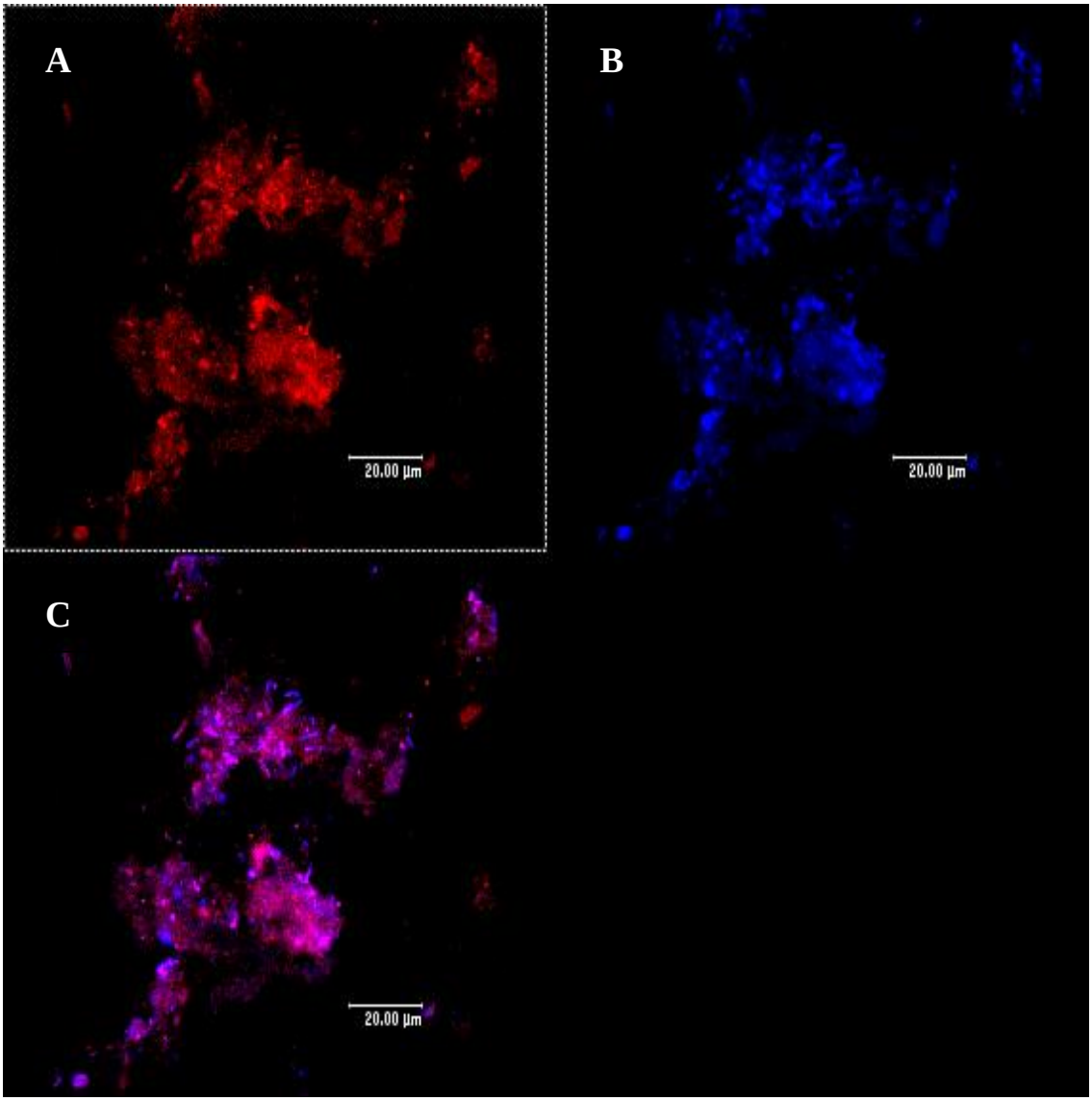


Figure 4.5. CLSM image of full-scale sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $MX825_{Cy5}$ (blue) probes (Scale bar, 20 µm).

As also seen in Figure 4.5, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* domain and the genus of *Methanoseate* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of the genus of *Methanoseate* within *Archaea* domain with dual hybridisation in one image (C). Sample was taken from mesophilic anaerobic digester at Hexham Sewage Treatment Works.

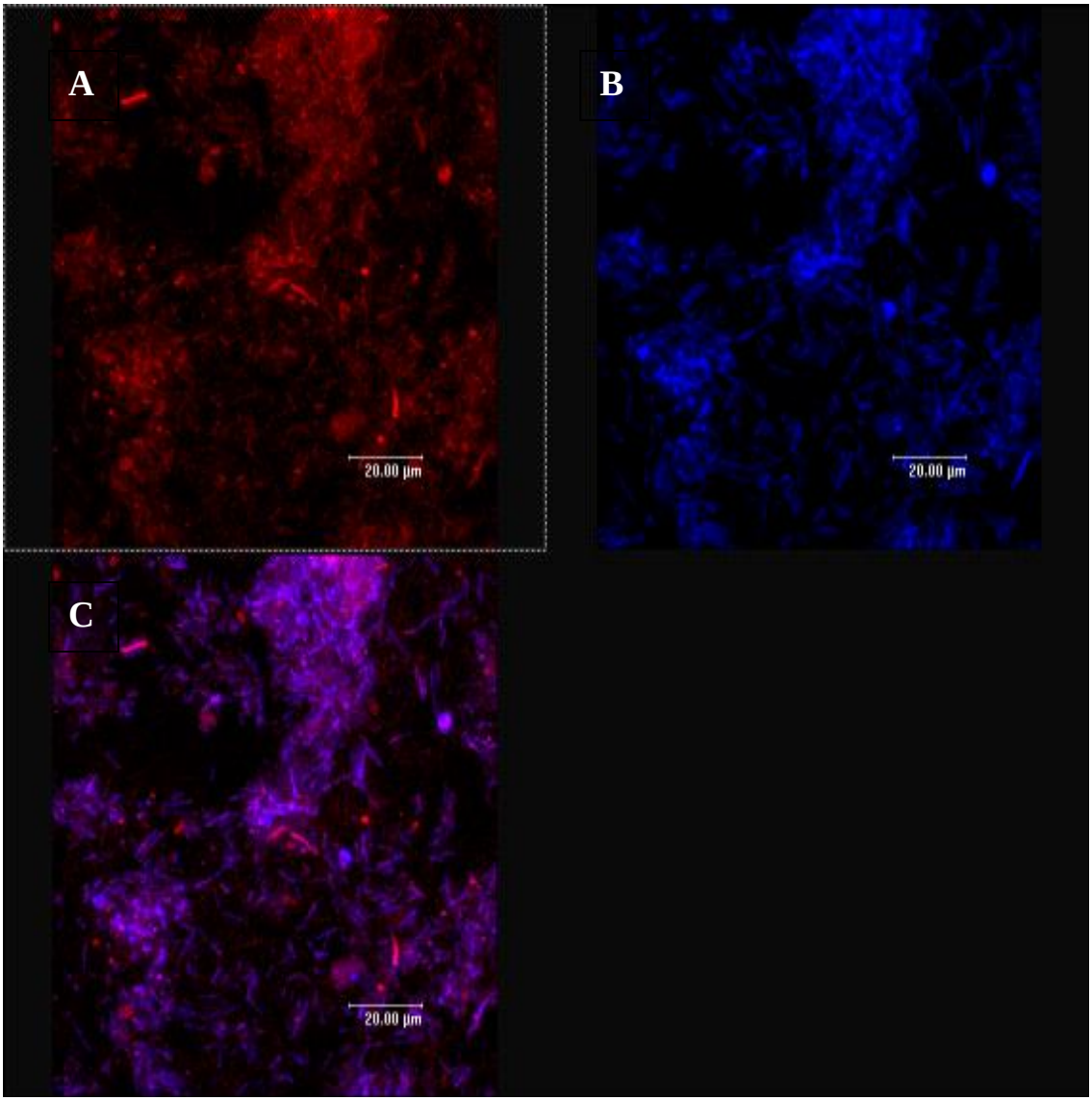


Figure 4.6. CLSM image of lab-scale sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $MX825_{Cy5}$ (blue) probes (Scale bar, 20 µm).

As also seen in Figure 4.6, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* domain and the genus of *Methanoseate* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of the genus of *Methanoseate* within *Archaea* domain with dual hybridisation in one image (C). Sample was taken from lab-scale anaerobic membrane bioreactor.

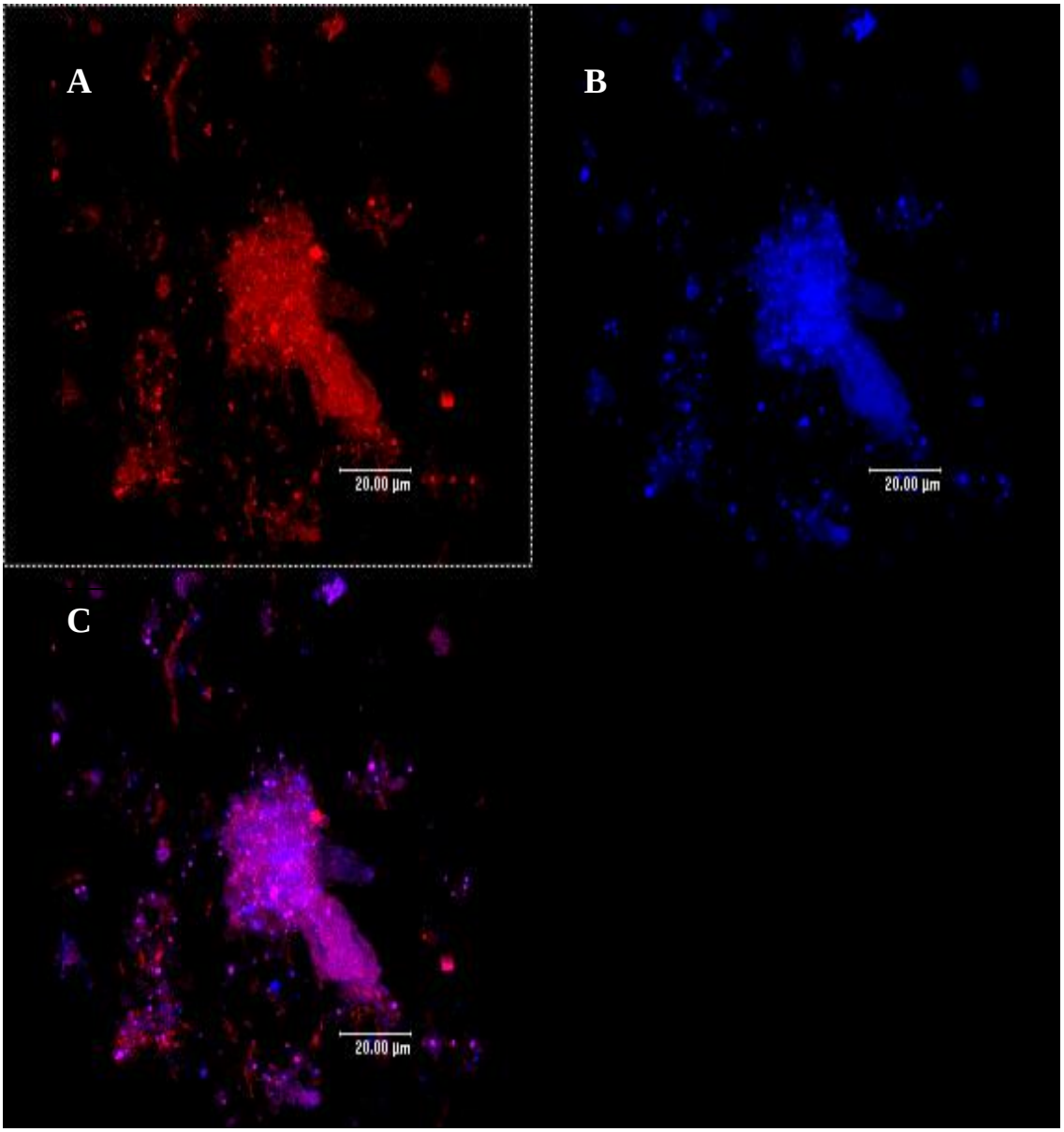


Figure 4.7. CLSM image of full-scale sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $MS821_{Cy5}$ (blue) probes (Scale bar, 20 µm).

As also seen in Figure 4.7, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* domain and the genus of *Methanosarcina* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of the genus of *Methanosarcina* within *Archaea* domain with dual hybridisation in one image (C). Sample was taken from mesophilic anaerobic digester at Hexham Sewage Treatment Works.

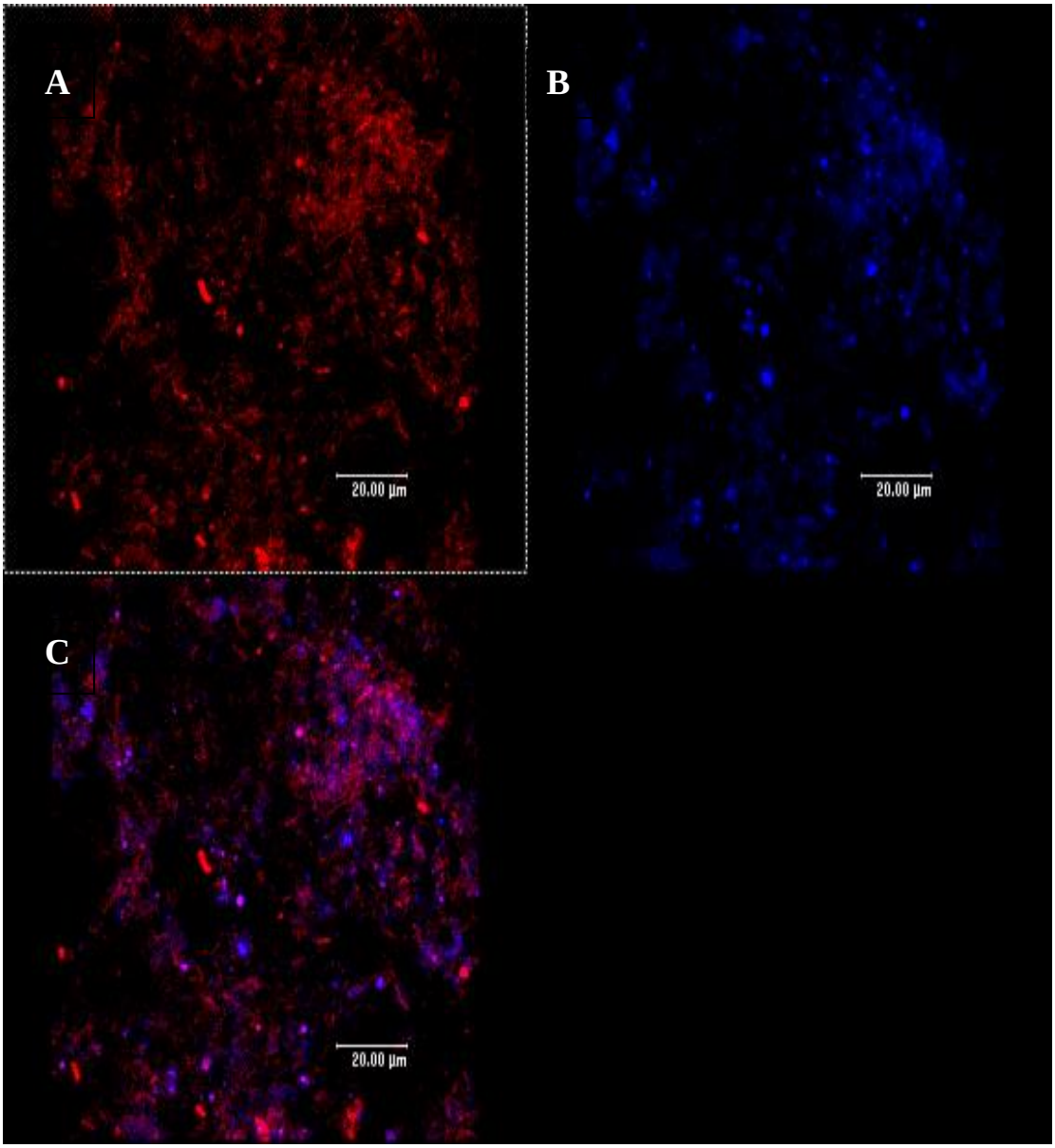


Figure 4.8. CLSM image of lab-scale sewage sludge sample dual hybridised with $Arc915_{Cy3}$ (red) and $MS821_{Cy5}$ (blue) probes (Scale bar, 20 µm).

As also seen in Figure 4.8, CLSM images were recorded seperately for each fluorescence emission channel in order to show abundance of *Archaea* domain and the genus of *Methanosarcina* in two different images (A, B). Furthermore, CLSM image was recorded sequentially for two channels in order to show abundance and localisation of the genus of *Methanosarcina* within *Archaea* domain with dual hybridisation in one image (C). Sample was taken from lab-scale anaerobic membrane bioreactor.

4.3. The Concentrations of Spesific Cells Obtained through Quantitative FISH Procedure

The numbers of *Archaea* , *Methanoseate*, *Methanosarcina* and *Bacteria* cells per milliliter was calculated after enumaration of the hybridised cells both for full scale and lab-scale anaerobic bioreactors. The variables demonstrated in Table 4.1. were used to calculate the numbers of cells per millilliter by quantitative FISH.

Table 4.1.The variables that contribute to the determination of the concentration of cells/unit volume by quantitative FISH

Full-scale digester	Cells/FOV (Mean Value)	Area of Sample (mm^2)	Total Area of FOV(mm^2)	Volume Sample (µl)	Dilution
Archaea Dual Hybridisation I	88.2	19.63	0.057	10	0.01
Archaea Dual Hybridisation II	92.8	19.63	0.057	10	0.01
Archaea Dual Hybridisation III	92.4	19.63	0.057	10	0.01
Methanoseate	41.6	19.63	0.057	10	0.01
Methanosarcina	42.2	19.63	0.057	10	0.01
Eubacteria	55.6	19.63	0.057	10	0.01
Lab-scale digester	Cells/FOV (Mean Value)	Area of Sample (mm^2)	Total Area of FOV(mm^2)	Volume Sample (µl)	Dilution
Archaea Dual Hybridisation I	199.8	19.63	0.0142	10	0.01
Archaea Dual Hybridisation II	193.6	19.63	0.0142	10	0.01
Archaea Dual Hybridisation III	179.8	19.63	0.0142	10	0.01
Methanoseate	128.6	19.63	0.0142	10	0.01
Methanosarcina	37.6	19.63	0.0142	10	0.01
Eubacteria	85.4	19.63	0.0142	10	0.01

Accordingly, the concentrations of *Archaea*, *Methanoseate*, *Methanosarcina* and *Eubacteria* are given in Table 4.2 for the samples of the full-scale and the lab-scale digesters, as also shown in Table 4.2.

Table 4.2. The number of spesific cells per ml for the samples taken from the full-scale and lab-scale anerobic digesters

Full-scale digester	*Archaea* cells per ml ($\times 10^9$)	*Methanoseate* cells per ml ($\times 10^9$)	*Methanosarcina* cells per ml ($\times 10^9$)	*Eubacteria* cells per ml ($\times 10^9$)
Dual Hybridisation I	1.52 ± 0.15	0.72 ± 0.12	-	-
Dual Hybridisation II	1.6 ± 0.15	-	0.73 ± 0.12	-
Dual Hybridisation III	1.59 ± 0.18	-	-	0.96 ± 0.13
Lab-scale digester	***Archaea* cells per ml ($\times 10^9$)**	***Methanoseate* cells per ml ($\times 10^9$)**	***Methanosarcina* cells per ml ($\times 10^9$)**	***Eubacteria* cells per ml ($\times 10^9$)**
Dual Hybridisation I	2.76 ± 0.18	1.78 ± 0.15	-	-
Dual Hybridisation II	2.68 ± 0.17	-	0.52 ± 0.14	-
Dual Hybridisation III	2.49 ± 0.15	-	-	1.18 ± 0.12

4.4. The Results of Analytical Process

Ratio of the variance (square of the standard deviation, or s^2) to the mean, called the index of dispersion (dispersion ratio) was calculated in order to determine how the *Archaea*, *Methanoseate*, *Methanosarcina* and *Eubacteria* are dispersed in the samples. The values of means, standard deviations, variances and index of dispersions for the samples were shown in Table 4.3. Distribution types determined according to the values of index of dispersion also exist in Table 4.3. F_{max} values of the samples were also shown in the tables. Critical F value was determined via statistical table given in appendix (Rohlf and Sokal, 1995).

In addition, the ratio of the largest variance to the smallest variance (F_{max}) was calculated and compared to the critical value from a known F_{max}- distribution in order to determine the homogeneity of variances within the *Archaea* cell counts for all three dual hybridisations. The critical value for three samples at four degrees of freedom is 15.5 at the 0.05 significance level. F_{max} = largest variance/smallest variance = 104.3 / 78.2 = 1.33 and F_{max} = largest variance/smallest variance = 176.7 / 124.7 = 1.42. As also seen in Table 4.3 (1a), the variances are homogenous due to the smaller F_{max} values of *Archaea* than critical value for the archaeal cells of the full-scale and the lab-scale digesters.

The Figure 4.9. demonstrates whether or not the data conform to normal distribution. Plotting frequency distribution curves and checking the normality with Anderson-Darling tests were achieved using standard statistical analysis software called MINITAB.

The y-axis represents the frequency of a particular observation and the x-axis is the count. Descriptive statistics are also given such as the mean, standard deviation and variance, and the results of a normality test are shown. The normal probability distributions were determined for the data compared to an expected normal distribution (Anderson-Darling Normality Test).

It has been found that all of the P values are greater than 0.05 which indicates that the data are normally distributed. However, the distribution of all archaeal cells from the full-scale digester are slightly skewed to the right (positive skewness values) and contains sharper peaks than normal curves (negative kurtosis values exist) as also demonstrated in the graphs of descriptive statistics.

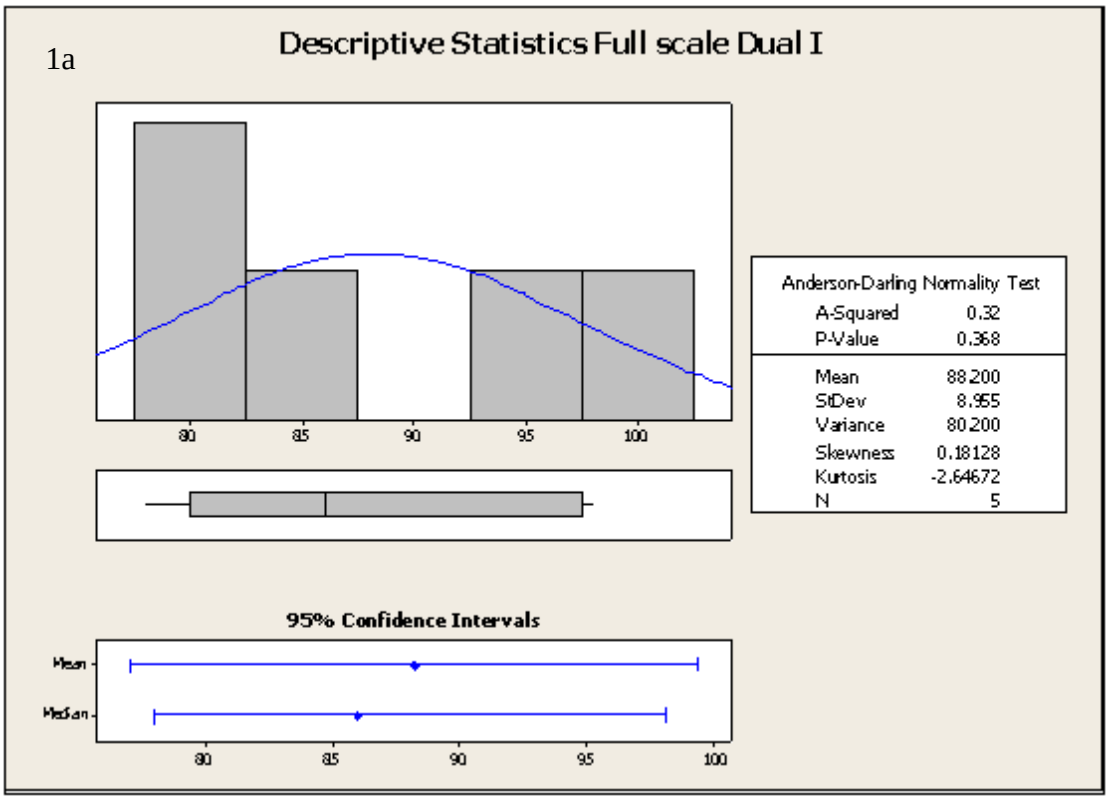
The frequency distributions with normality curve, descriptive statistics and normal probability distributions of all other cells present in the samples are given in appendix. As also seen in appendix, the distributions of all other cells are roughly similar, except the archaeal cells in dual hybridisation II and dual hybridisation III for the sample of the lab-scale digester.

Table 4.3. Determination of the index of dispersion from estimates of the mean and variance, for counts of 1a) *Archaea*, 1b) *Methanoseate*, *Methanosarcina* and *Eubacteria* in the samples of full-scale and lab-scale anaerobic digestors using FISH.

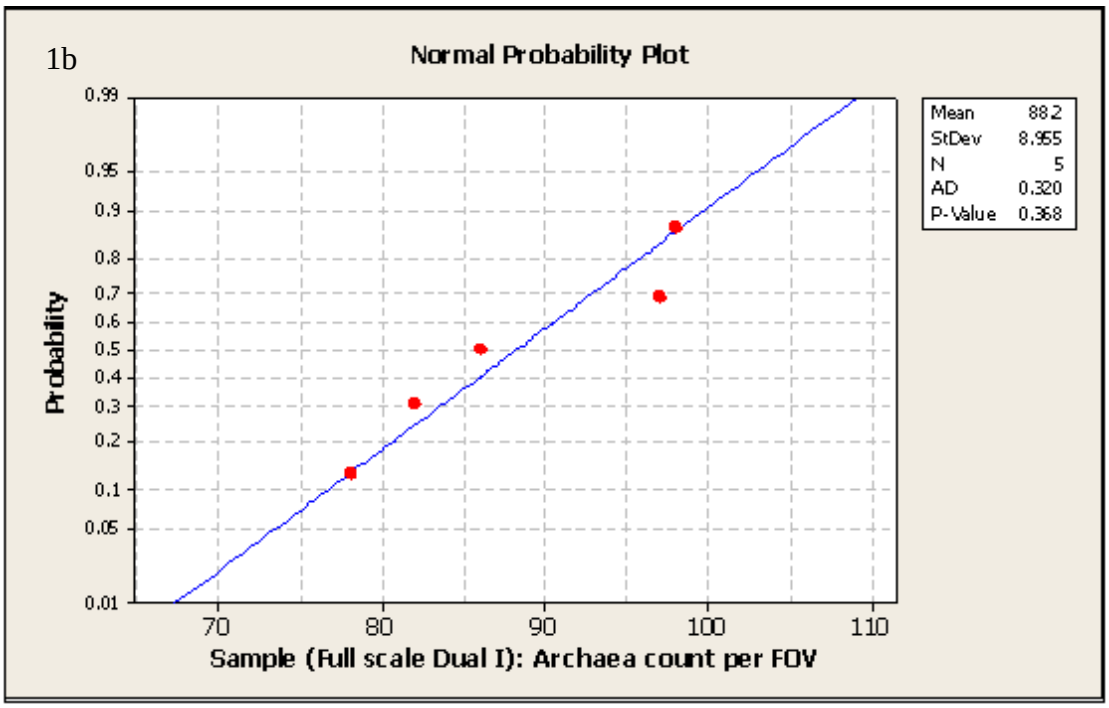
1a	Number of Archaea cells per field of view (FOV)					
Sample	Full scale digester			Lab-scale digester		
Subsample	Dual Hyb I	Dual Hyb II	Dual Hyb III	Dual Hyb I	Dual Hyb II	Dual Hyb III
	86	100	91	204	198	183
	97	83	79	184	179	165
	78	93	98	189	182	174
	98	85	106	205	199	182
	82	103	88	217	210	195
Mean (x^m)	88.2	92.8	92.4	199.8	193.6	179.8
S.D. (s)	8.955	8.843	10.213	13.29	12.90	11.17
Variance (s^2)	80.2	78.2	104.3	176.7	166.3	124.7
Dispersion Ratio	**0.91**	**0.84**	**1.1**	**0.88**	**0.86**	**0.7**
Distribution type	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)
Fmax value	**1.33**			**1.42**		
Critical F value	**15.5**					

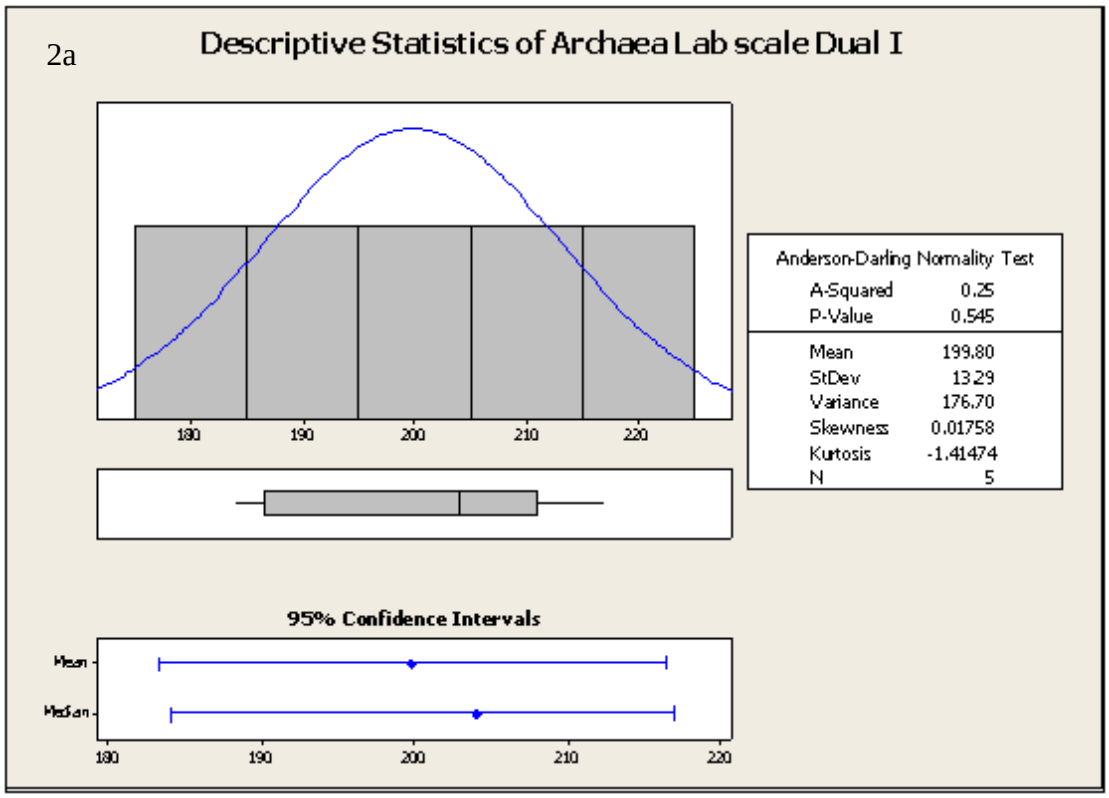
1b	Number of *Methanoseate* cells per field of view (FOV)		Number of *Methanosarcina* cells per field of view (FOV)		Number of *Eubacteria* cells per field of view (FOV)	
Sample	Full-scale digester	Lab-scale digester	Full-scale digester	Lab-scale digester	Full-scale digester	Lab-scale digester
	47	132	53	32	46	75
	40	142	42	27	52	80
	38	119	33	41	61	93
	50	117	36	38	54	84
	33	133	47	50	65	95
Mean (x^m)	41.6	128.6	42.2	37.6	55.6	85.4
S.D. (s)	6.88	10.45	8.106	8.79	7.5	8.5
Variance (s^2)	47.3	109.3	65.2	77.3	56.3	72.3
Dispersion Ratio	**1.14**	**0.85**	**1.6**	**2.06**	**1.01**	**0.85**
Distribution type	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)	Negative binominal distribution (for contagious dispersion)	Negative binominal distribution (for contagious dispersion)	Poisson distribution (for random dispersion)	Poisson distribution (for random dispersion)

1a


Descriptive Statistics Full scale Dual I
80
85
90
95
100
Anderson-Darling Normality Test
A-Squared 0.32
P-Value 0.368
Mean 88.200
StDev 8.955
Variance 80.200
Skewness 0.18128
Kurtosis -2.64672
N 5
95% Confidence Intervals
Mean
Median
80
85
90
95
100

1b


Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
70
80
90
100
110
Sample (Full scale Dual I): Archaea count per FOV
Mean 88.2
StDev 8.955
N 5
AD 0.320
P-Value 0.368



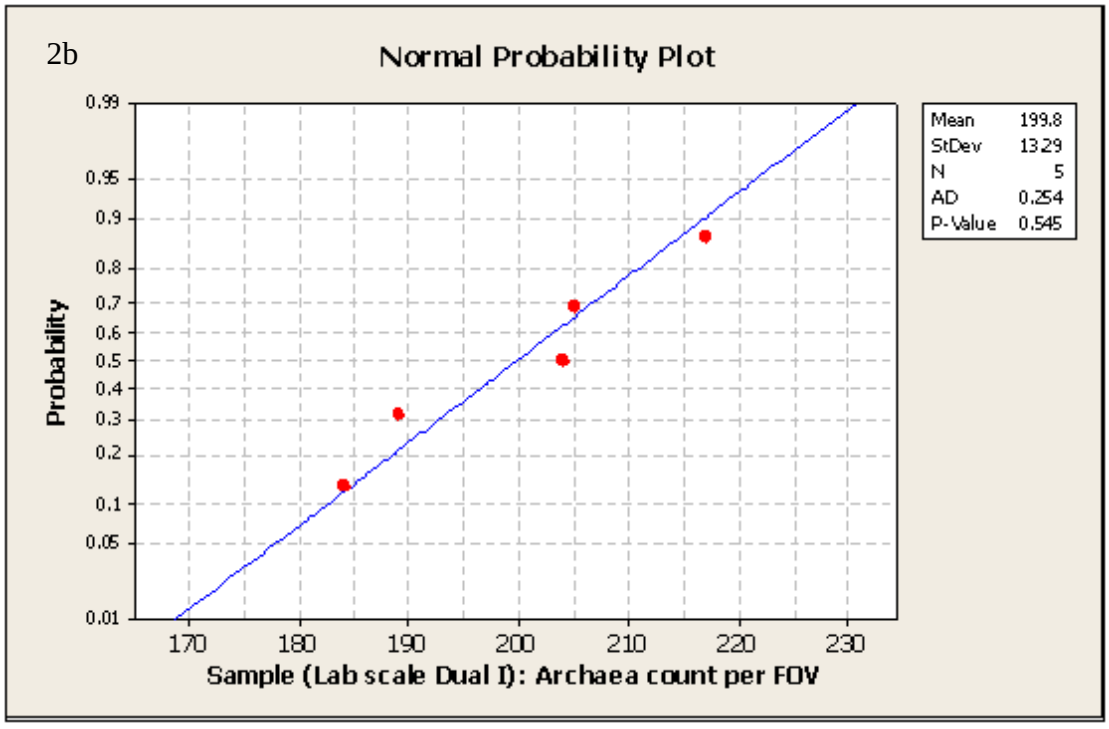


Figure 4.9. Typical MINITAB outputs for: Frequency distributions with normality curve, descriptive statistics and normal probability distributions for archaeal cells present in the full-scale (1a, 1b) and in the lab-scale (2a, 2b) digesters.

4.5. The Box-Cox plots

The Box-Cox plots were obtained through MINITAB statistical software program in order to determine the transformations required for normal distribution. Figure 4.10 demonstrates the Box-Cox plots as MINITAB outputs for the archaeal cells in the samples.

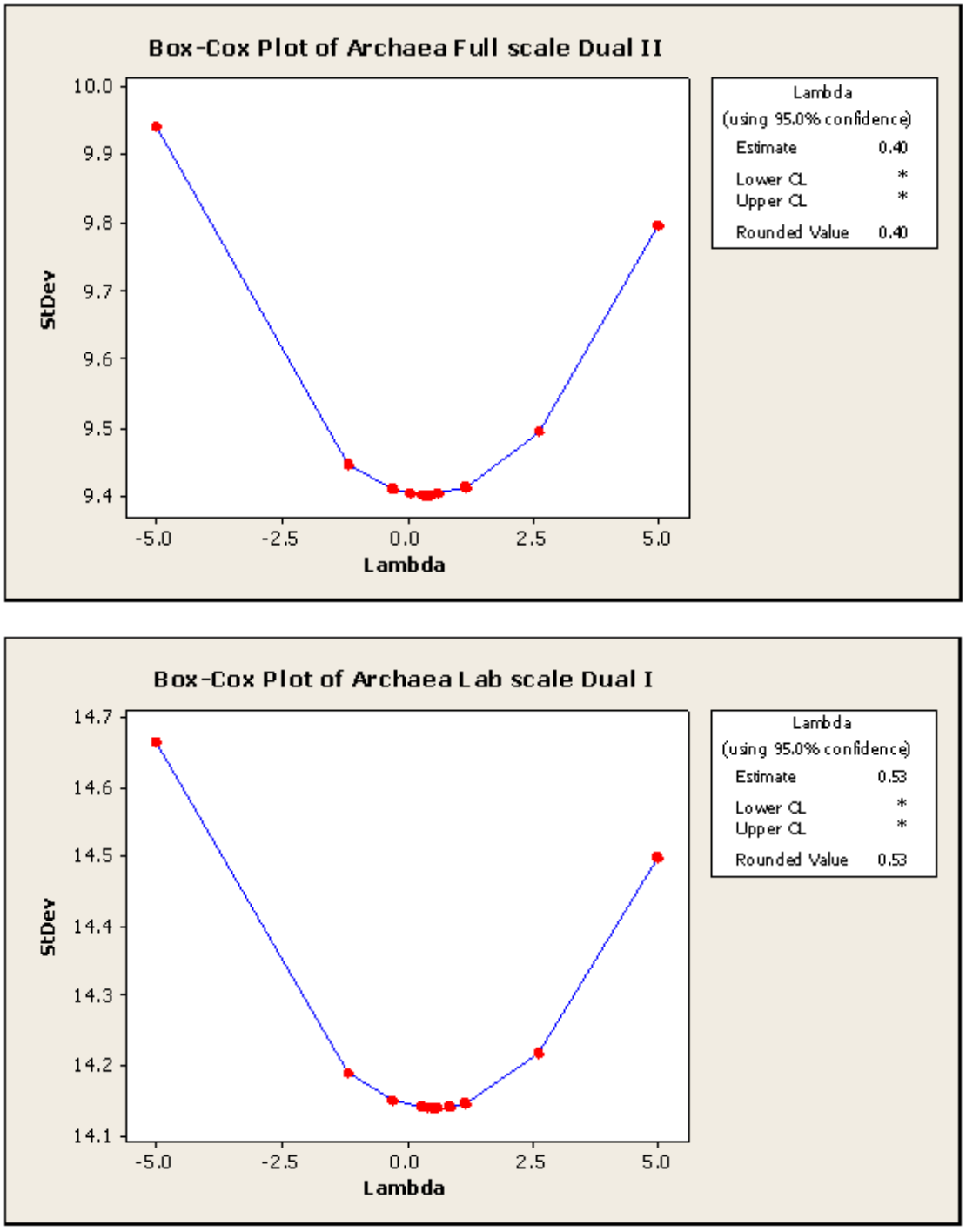


Figure 4.10. Box-Cox plots as MINITAB outputs for archaeal cells present in the samples taken from the full-scale and the lab-scale anaerobic digesters.

λ-values determined through Box-Cox plots indicates the nature of transformation required to obtain normally distributed data. The λ-value of approximately 0.5 for the archaeal cells in the anaerobic digesters indicates that a SQRT transformation is appropriate. The box-cox plots of other cells present in the samples are given in appendix. Accordingly, the λ-values of approximately 0.5 for the *Methanoseate* and *Eubacteria* cells, indicate that a SQRT transformation is appropriate, further the λ-value of approximately 0 for the *Methanosarcina* cells, indicates that a logarithmic transformation is appropriate.

4.6. One-way Analysis of variance (ANOVA)

The results of one-way ANOVA for archaeal cells are shown in Table 4.4. The results indicate that the means of the two samples taken from full-scale and lab-scale digesters are highly statistically significant ($P < 0.0001$).

Table 4.4. The abundance data of 1a) the archaeal cells in the samples following FISH. 1b) compared by ANOVA and a multiple comparison of means using MINITAB.

1a

	Archaea in lab scale anaerobic digester			*Archaea* in full scale anaerobic digester		
Sampling unit, n (FOV)	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml
1	204	2.82	53104	100	1.72	41496
2	184	2.54	50434	83	1.43	38804
3	189	2.61	51115	93	1.6	40017
4	205	2.83	53235	85	1.46	38258
5	217	2.99	54761	103	1.77	42114
Mean, x^m	-	2.76	**52530**	-	1.6	**40138**
S.D., s	-	0.184	1746	-	0.15	1664
Coefficient of variation, *CV* (%)	-	6.65	3.33	-	9.53	4.77

1b

One-Way Analysis of Variance : SQRT Archaea conc. Lab-scale, SQRT Archaea conc. Full scale

```
Source  DF         SS         MS       F      P
Factor   1  383904160  383904160  131.94  0.000
Error    8   23276640    2909580
Total    9  407180800

S = 1706   R-Sq = 94.28%   R-Sq(adj) = 93.57%


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level           N   Mean  StDev  ----+---------+---------+---------+-----
SQRT Archaea L  5  52530   1746                                  (---*----)
SQRT Archaea F  5  40138   1664  (---*----)
                                 ----+---------+---------+---------+-----
                                 40000     44000     48000     52000

Pooled StDev = 1706


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


SQRT Archaea L subtracted from:

                Lower  Center  Upper
SQRT Archaea F  -14880  -12392  -9904

                     +---------+---------+---------+---------
SQRT Archaea F       (----*----)
                     +---------+---------+---------+---------
                   -15000    -10000     -5000         0
```

The results of one way ANOVA for other cells (*Methanoseate*, *Methanosarcina* and *Eubacteria*) are given in appendix. As also seen in appendix, the P values of 0 obtained with one-way analyses of variance indicate the statistical differences between the samples.

CHAPTER 5. DISCUSSION

The quantitative fluorescent *in situ* hybridisation is a practible technique for detection and enumeration of microorganisms taking place within the anaerobic sludge samples. Moter and Göbel (2000) also stated that as a technique allowing simultaneous visualization, identification, enumeration and localization of individual microbial cells, fluorescence *in situ* hybridization (FISH) is useful for many applications in all fields of microbiology. However, there exists some methodological limitations such as the flaws of fluorochrome and difficulties in the adjustments of stringency conditions.

The cyanine series Cy has taken the place of most commonly used fluorochromes namely, fluorescein and rhodamine-derivatives (FITC, FLUOS and TRITC) in the mid of nineties. This replacement is due to the some limitations of common fluorochromes such as broad emission band and low photostability. These disadvantages result in less convenience for quantitative multifluorescence microscopy. In other words, the flaws of fluorophores effect dual staining negatively. The cyanine series are superior to the classical fluorescein dyes, because they result in significantly brighter staining and are very stable to photobleaching (Wessendorf and Brelje, 1992; Manz *et al*., 1998). Therefore, two fluorophores from the cyanine series namely, Cy3 and Cy5 were used for the labelling of the *Archaea* domain- (ARC915) and two methanogenic archaeal genera- spesific probes (MX825 and MS821) in the present study. FITC was also used as the fluorophore of the *Bacteria* domain-spesific probe mixture (EUB338 I, II and III).

EUB338I is generally used for positive control. However, Daims *et. al* (1999) indicated that some *Bacteria* can not be detected by only this probe. In addition, Moter and Göbel (2000) suggested that the use a set of bacterial probes might provide more accurate analysis for complex microbial communities, hence EUB338I, EUB338II and EUB338III were used as a mixture in order to detect all *Bacteria* in the sludge samples taken from the anaerobic digesters for this study.

On the other hand, hybridisation depends on the stringent conditions to provide spesific binding of probe to the corresponding target sequence. Stringency was

regulated by means of variations in temperature, denaturant namely formamide in hybridisation step and salt concentration in washing step for each hybridisation process. The short oligonucleotides hybridise less specifically with any target sequence at low temperatures. However, formamide decreases melting point of duplex-structure (probe and target) by weakening the hydrogen bonds, enabling lower temperatures to be used with high stringency (Moter and Göbel, 2000). Formamide also helps soften RNA structure, allowing better hybridisations. Under the light of this knowledge, melting temperatures were kept roughly similar by adjusting formamide concentrations due to the use of multiple probes for each sample, further post hybridisation stringent conditions were supplied with the variation of salt concentration in washing step.

Dual hybridisation combined with confocal scanning microscopy is an appropriate way of multi-target visualisation and quantitative analysis for the samples taken from the anaerobic digesters. Confocal microscopy detects structures by collecting light from a single focal plane of the sample, excluding light that is out of focus. The microscope lenses focus the laser light on one point in the specimen. The laser moves rapidly from point to point to produce the scanned image (Data from user manual of Leica TCS SP2). The effect of elimination of out-of-focus light depends on the numerical aperture of the microscope lens and size of the pinhole (Cited in Wagner *et al*., 1998). Therefore, the settings of confocal scanning microscope were optimised in order to obtain images with high resolution.

In anaerobic treatment systems, two important genera namely, *Methanoseate* and *Methanosarcina* are responsible for acetoclastic reaction producing two thirds of methane from acetate. The ratios of *Methanoseate* to *Methanosarcina* are different for the samples taken from full-scale anaerobic digester and lab-scale anaerobic membrane bioreactor , although the lab-scale digester had been seeded with the same full-scale sludge. This finding confirmed the previous studies. For instance, Casserly and Erijman (2003) suggests that the initial inoculum does not determine the structure of the microbial composition at later steps of operation.

According to the results obtained with DAPI staining in the present study, the concentrations of whole cell taking place in the full-scale conventional anaerobic digester and lab-scale anaerobic membrane bioreactor are $3.19 \pm 0.5 \times 10^9$ cells ml^{-1} and $4.03 \pm 0.53 \times 10^9$ cells ml^{-1}, respectively. The total cells per milliliter for each

sample were enumerated in order to determine the proportion of two different genera of acetoclastic methanogens.

Three dual hybridisations were carried out using *Archaea Methanoseate*, *Methanosarcina* and *Eubacteria* spesific probes for each anaerobic sample. The concentrations of archaeal cells present in the sample of full-scale digester are $1.52 \pm 0.15 \times 10^9$ cells ml^{-1}, $1.6 \pm 0.15 \times 10^9$ cells ml^{-1} and $1.59 \pm 0.18 \times 10^9$ cells ml^{-1} for dual hybridisation I, II and III. The concentrations of *Archaea* for the sample of lab-scale digester are $2.76 \pm 0.18 \times 10^9$ cells ml^{-1}, $2.68 \pm 0.17 \times 10^9$ cells ml^{-1} and $2.49 \pm 0.15 \times 10^9$ cells ml^{-1} for each dual hybridisation. These concentrations calculated by means of quantitative FISH procedure confirmed that the archaeal cell counts per ml in each sample are similar for all of the dual hybridisations. Moreover, variances of archaeal population between the dual hybridisations for both of the samples were determined as homogeneous according to the statistical analysis. These findings are expected due to the use of the same *Archaea* spesific probe in three different dual hybridisations carried out for the same sample in this study.

Besides, the concentrations of *Methanoseate* are $7.2 \pm 1.2 \times 10^8$ cells ml^{-1} and $1.78 \pm 0.15 \times 10^9$ cells ml^{-1} , the concentrations of *Methanosarcina* are $7.3 \pm 1.2 \times 10^8$ cells ml^{-1} and $5.2 \pm 1.4 \times 10^8$ cells ml^{-1} and the concentrations of *Eubacteria* are $9.6 \pm 1.3 \times 10^8$ cells ml^{-1} and $1.18 \pm 0.12 \times 10^9$ cells ml^{-1} for the samples of full-scale and lab-scale digesters. These outcomes indicate that there exist differences between the samples in terms of the concentrations of *Archaea*, *Methanoseate*, *Methanosarcina* and *Eubacteria*. The comparisons of the numbers of cells per milliliter have also been demonstrated in Figure 5.1.

Table 5.1 also summarizes the average concentrations of DAPI-stained and hybridised cells with oligonucleotide probes for each sample.

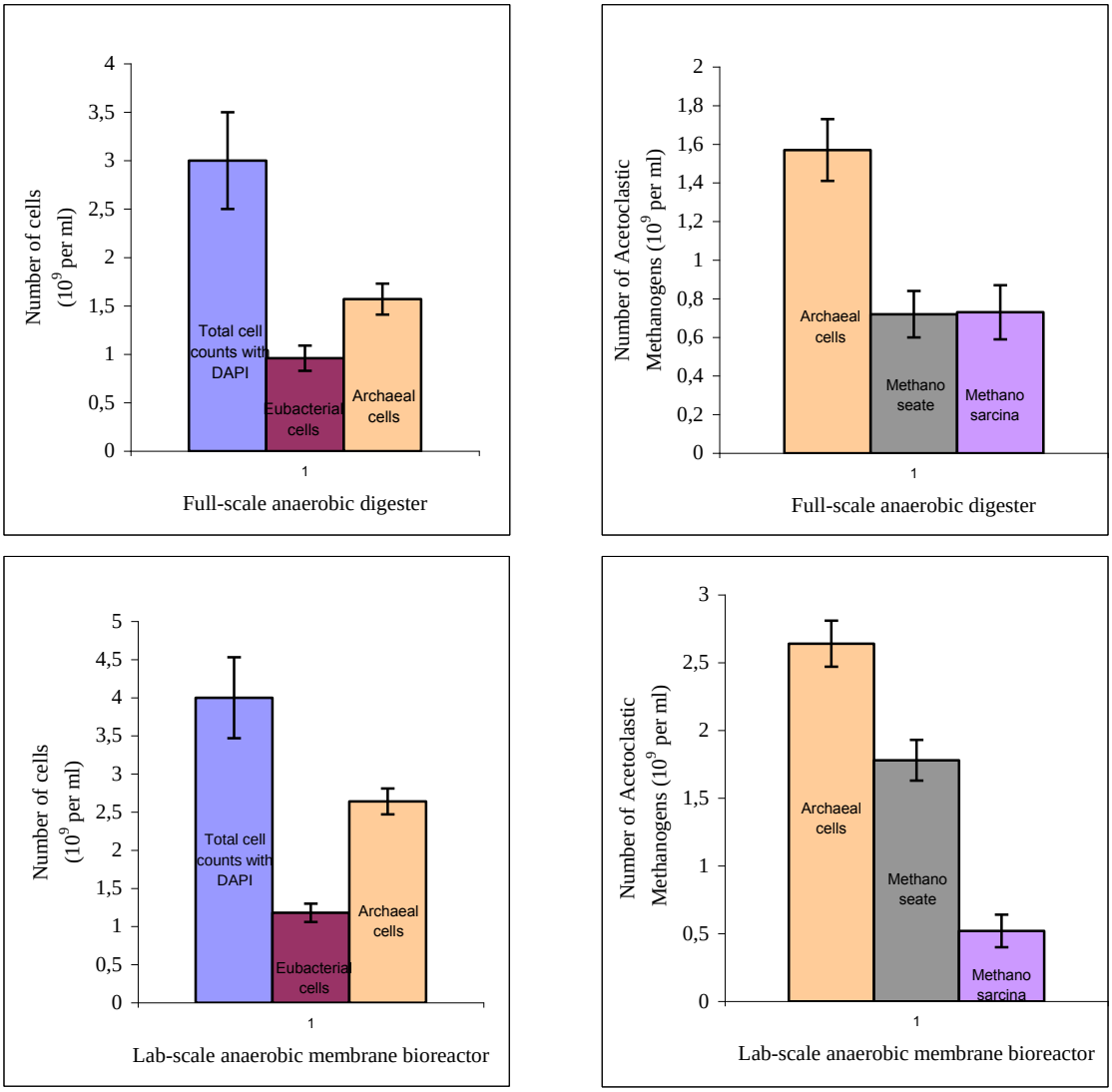


Figure 5.1. Comparisons of the numbers of total cell, *Archaea* (ARC915), *Methanoseate* (MX825), *Methanosarcina* (MS821) and *Eubacteria* ($EUB338_{mix}$) determined by DAPI staining and FISH for the samples taken from full-scale and lab-scale anaerobic bioreactors.The error bars indicate the standard deviations.

On the other hand, the ratio of *Methanoseate* to *Methanosarcina* for the sample of lab-scale digester is high due to the configuration type of reactor. Wen *et al*. (1999) stated that sufficient biomass will ensure good performance in COD removal and better quality effluent. Anaerobic membrane bioreactor used in this study provided an effective retention of biomass within the bioreactor with a membrane. Therefore, this experimental anaerobic treatment system has the ability of 98% COD removal efficiency.

Table 5.1. Average numbers of DAPI-stained cells and cells after hybridisation with oligonucleotide probes

	Average DAPI-stained cells (cells/ml)*	
Stain	In full-scale digester	In lab-scale digester
DAPI	$3.19 \pm 0.5 \times 10^9$	$4.03 \pm 0.53 \times 10^9$
	Average hybridised cells (cells/ml)**	
Probe	In full-scale digester	In lab-scale digester
ARC915	$1.6 \pm 0.15 \times 10^9$	$2.76 \pm 0.18 \times 10^9$
MX825	$7.2 \pm 1.2 \times 10^8$	$1.78 \pm 0.15 \times 10^9$
MS821	$5.2 \pm 1.4 \times 10^8$	$7.3 \pm 1.2 \times 10^8$
$EUB338_{mix}$	$9.6 \pm 1.3 \times 10^8$	$1.18 \pm 0.12 \times 10^9$

* Average number of DAPI-stained cells ± S.D. (n=20)

** Average number of cells hybridised with probes ± S.D. (n=5)

Fukuzaki and his coworkers (1990) denoted that the kinetics of acetate utilization by digestor sludge would depend on the predominant species in the population of acetoclastic methanogens. *Methanoseate* has lower growth rate than *Methanosarcina* at high acetate concentrations. However, the affinity of *Methanoseate* for acetate is much higher than *Methanosarcina*. This is due to the fact that acetate is the only growth substrate for *Methanoseate*.

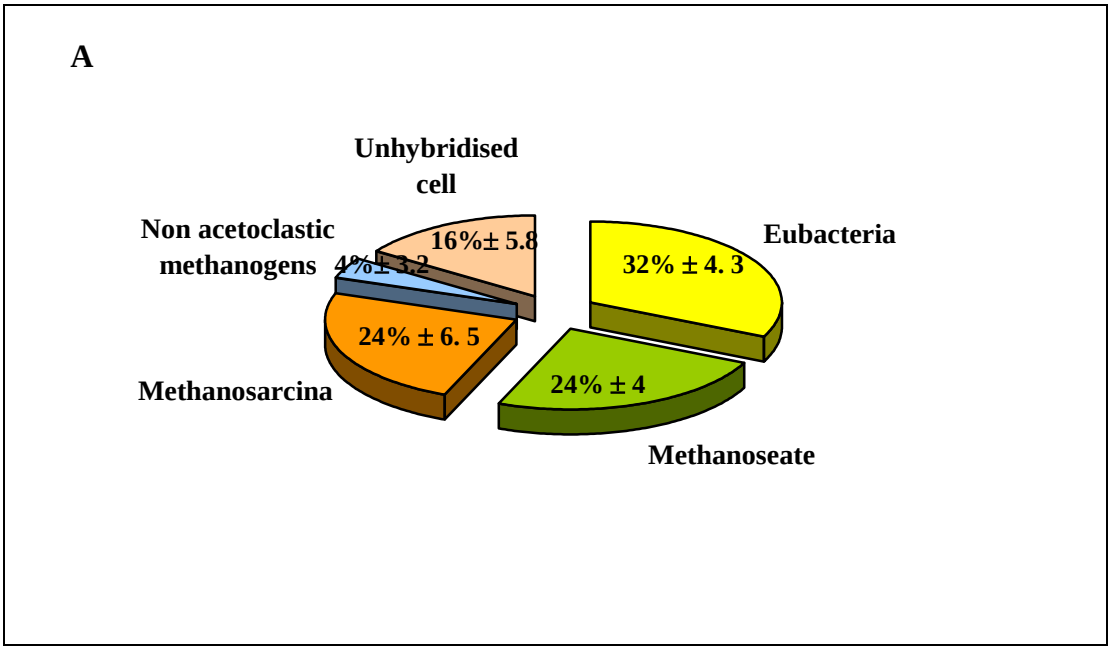
The outstanding predominance of Methanoseate exists in the lab-scale reactor. For instance, Anderson and his colleagues (2003) proved that *Methanoseate* will be the dominant acetoclastic species at acetate concentrations below 1mM. Accordingly, it has been understood that 0.15mM (8.75 mg/l) of acetate concentration could enable the majority of *Methanoseate* within the lab-scale reactor.

The existence of *Methanoseate* species in the reactor depends on the operational conditions such as pH and temperature. There exist one mesophilic and one thermophilic species belonging to the genus *Methanoseate* namely, *Methanoseate concilii* and *Methanoseate thermophila*. The pH value of the lab-scale reactor is 6.84 and the reactor was operated in the mesophilic range of temperature. This information demonstrated that *Methanoseate concilii* which is mesophilic species could merely survive in this reactor.

The ratio of *Methanoseate* to *Methanosarcina* approximately equals to one in the full-scale reactor due to the similarity between the concentration values of *Methanoseate* and *Methanosarcina*.

The pH value of the full-scale anaerobic digestor used for sampling is 6.9, further this system is also operated in the mesophilic range of temperature like lab-scale anaerobic membrane bioreactor. There exist five mesophilic and only one thermophilic species belonging to the genus *Methanosarcina* namely, *M. barkeri*, *M. acetivorans*, *M. mazeii*, *M. siciliae*, *M. vacuolata* and *M. thermophila*. However, it might be mentioned about the existence of only *M. barkeri*, *M. mazeii* and *M. vacuolata* within the full-scale conventional anaerobic digester due to the pH and temperature values of the reactor.

The concentrations of cells obtained with the quantitative FISH procedure have been transformed to their percentage values. The percentages of 16S rRNAs of cells present in the samples has been illustrated through the pie charts in Figure 5.2. This figure indicates the microbial composition difference between the samples of full-scale and lab-scale digesters. Furthermore, the statistical analysis performed for this study confirmed that the samples are statistically different from each other.



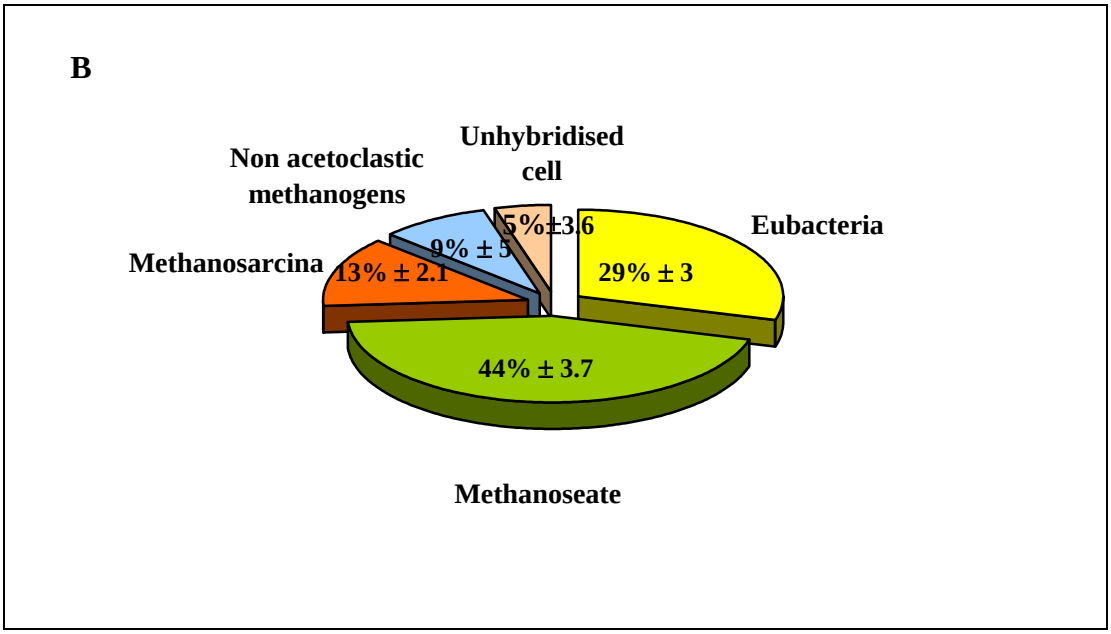


Figure 5.2. Pie charts showing the percentages of 16S rRNAs of *Eubacteria* ($EUB338_{mix}$), *Archaea* (ARC915) and of two acetoclastic methanogenic genera, *Methanoseate* (MX825) and *Methanosarcina* (MS821) present in the samples of full-scale (A) and lab-scale (B) anaerobic digesters.

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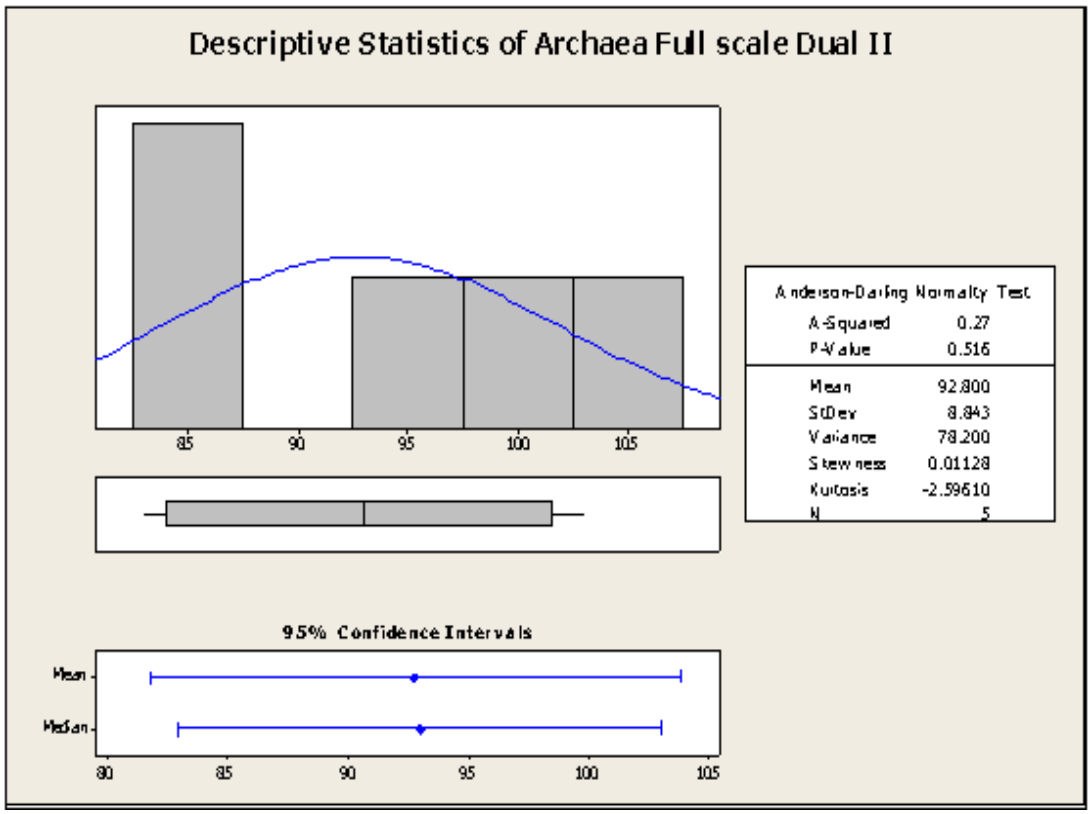
APPENDIX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Variance 78.200
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Kurtosis -2.59610
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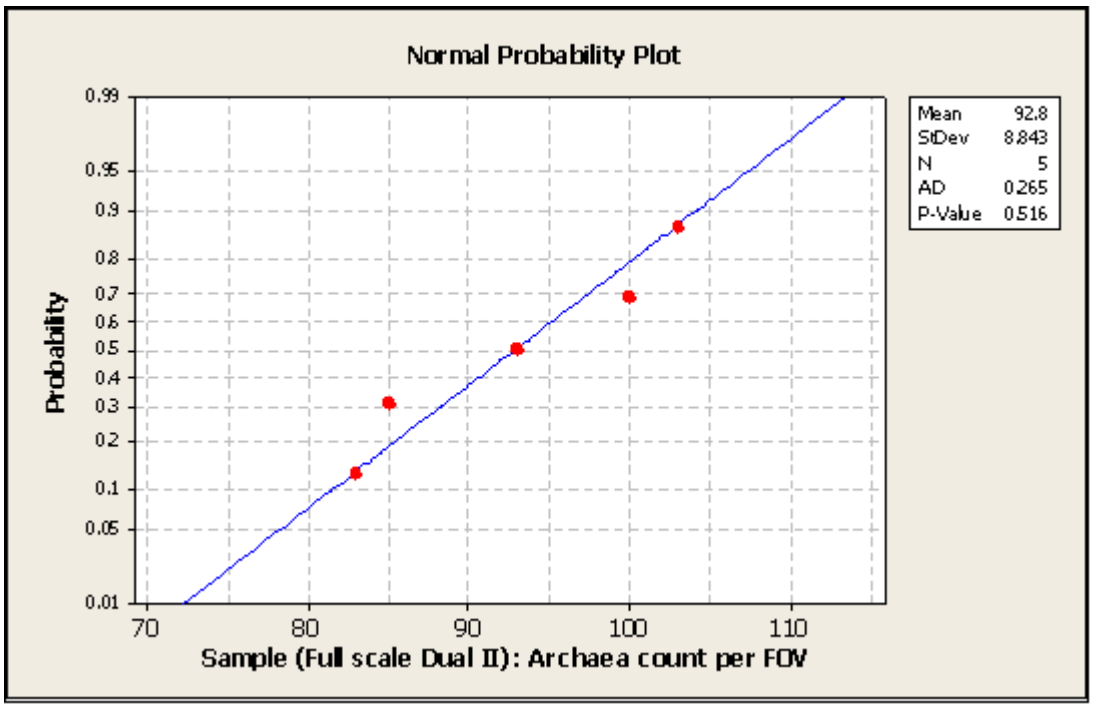
Mean
Median
80
85
90
95
100
105


Normal Probability Plot
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0.4
0.3
0.2
0.1
0.05
0.01
70
80
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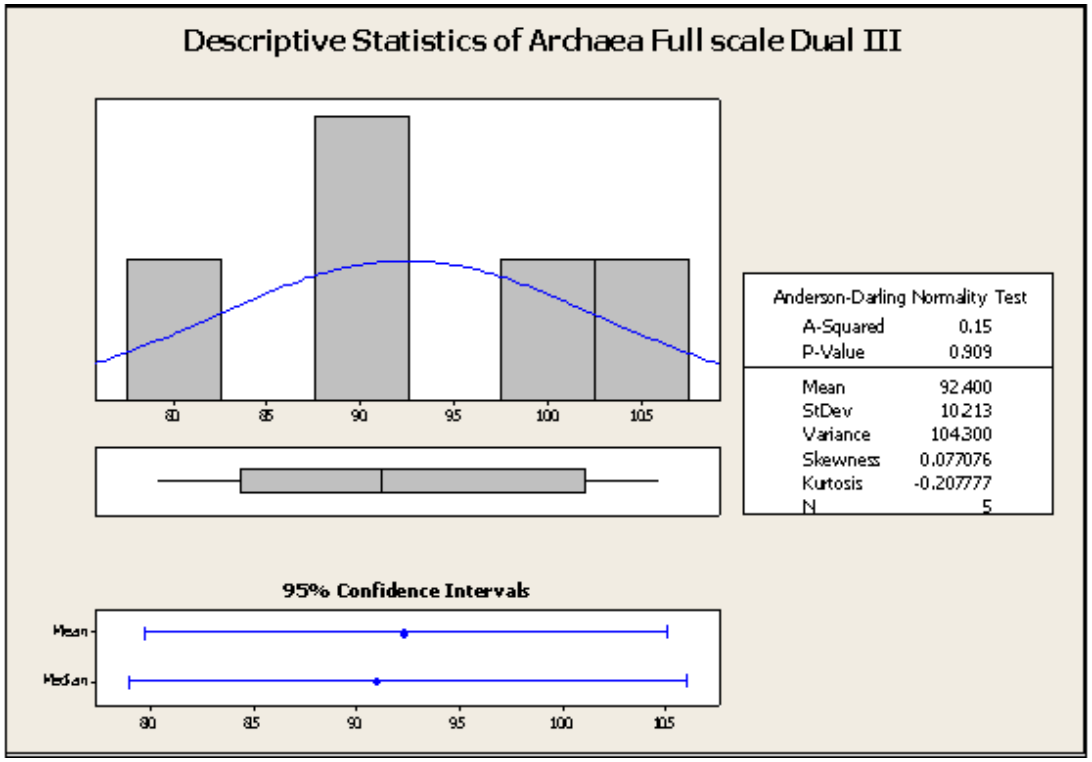
100
110
Sample (Full scale Dual II): Archaea count per FOV
Mean 92.8
StDev 8.843
N 5
AD 0.265
P-Value 0.516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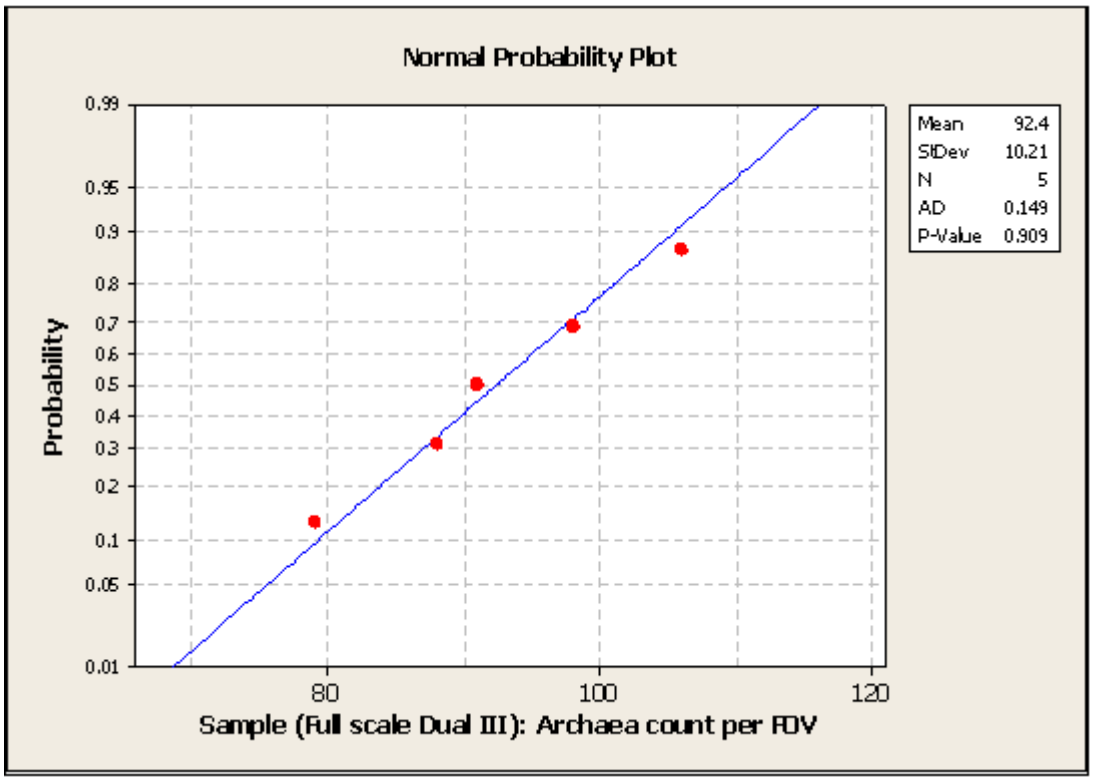
N 5
95% Confidence Intervals
Mean
MeSan


Normal Probability Plot
Probability
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0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
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100
120
Sample (Full scale Dual III): Archaea count per FOV
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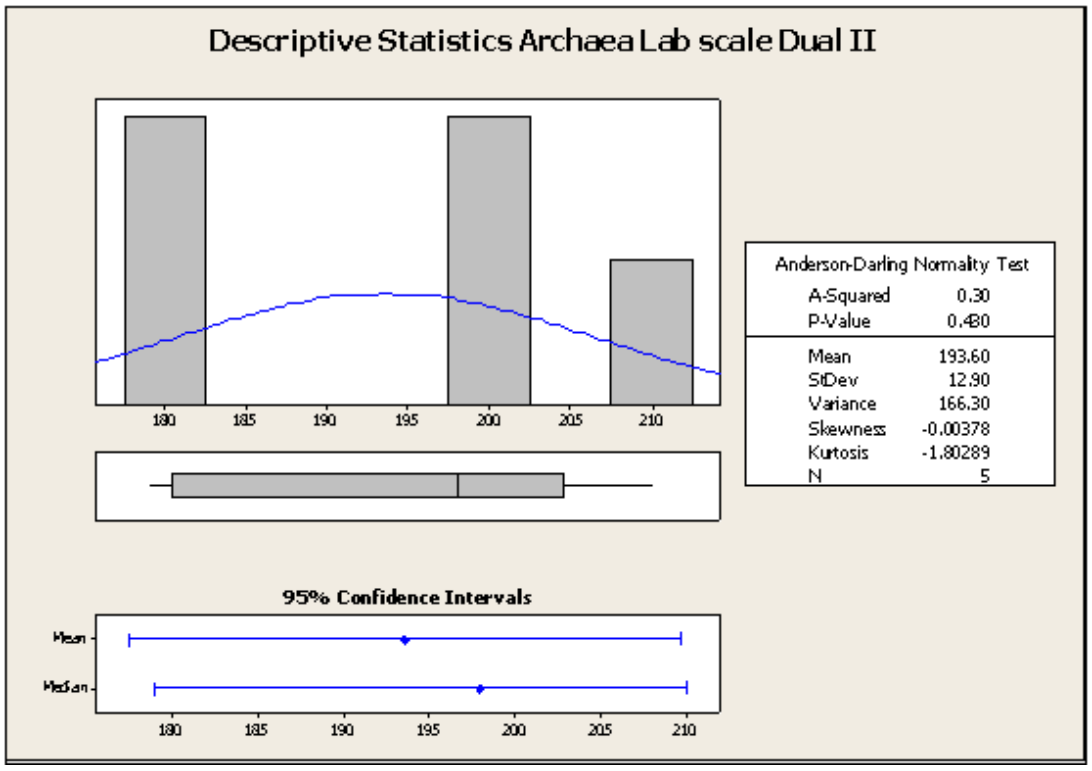
StDev 10.21
N 5
AD 0.149
P-Value 0.909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Kurtosis -1.80289
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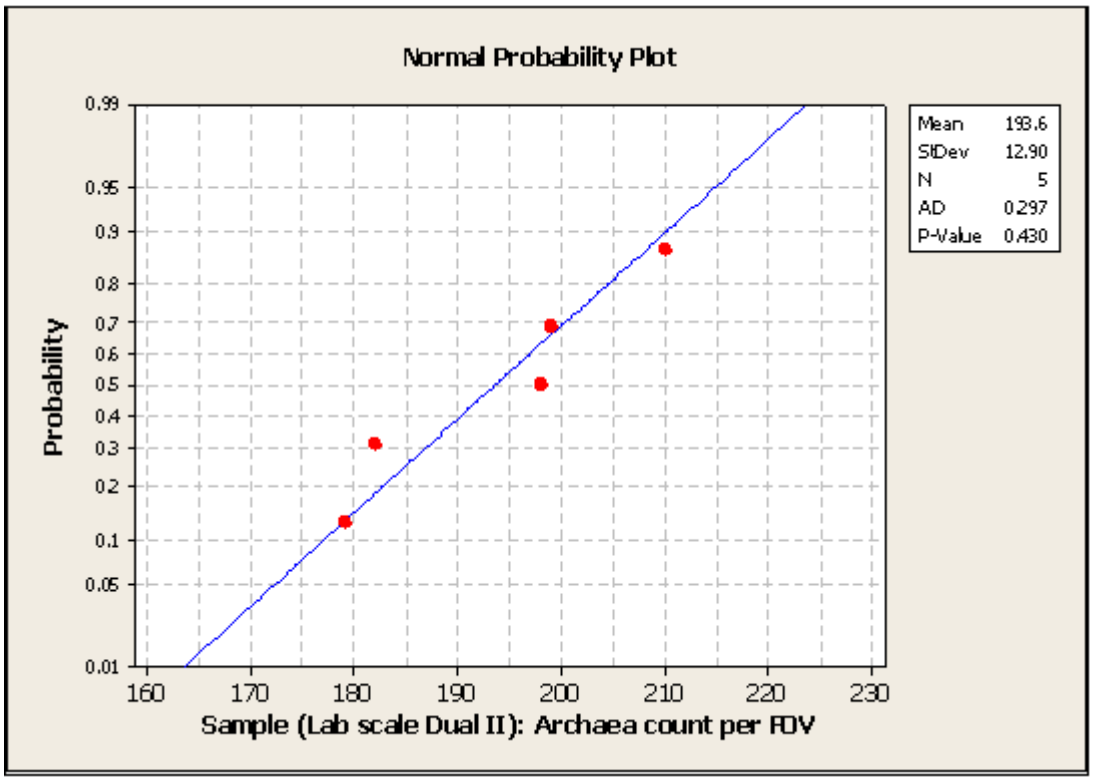
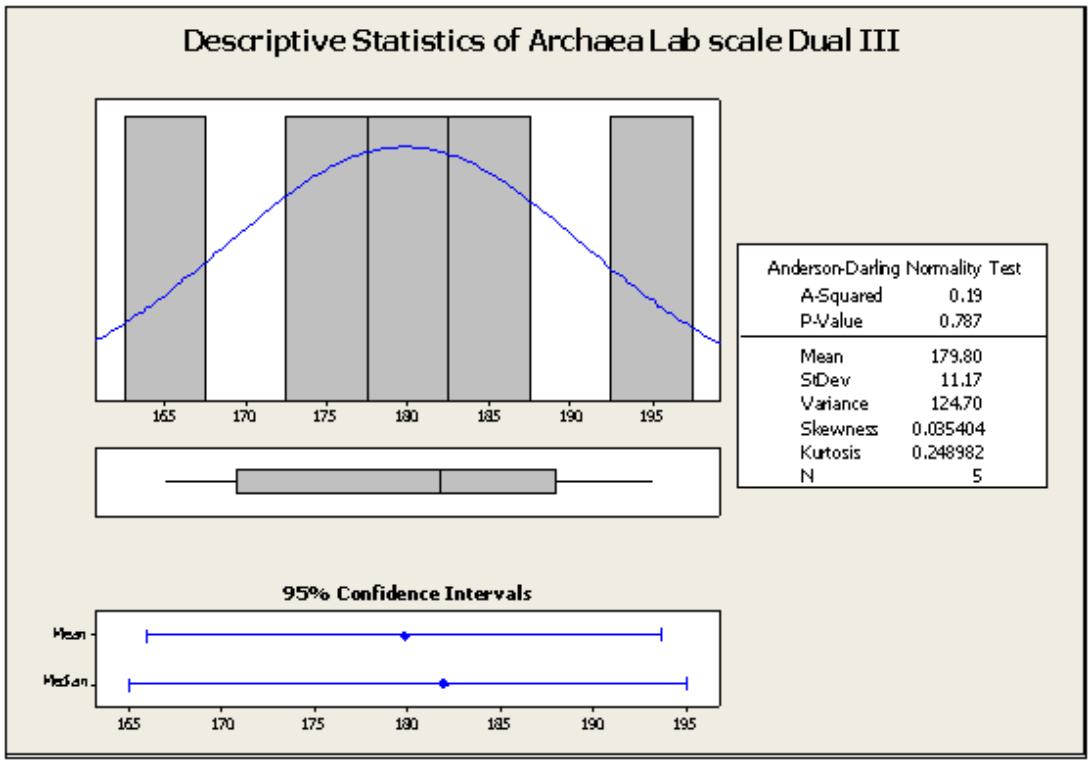
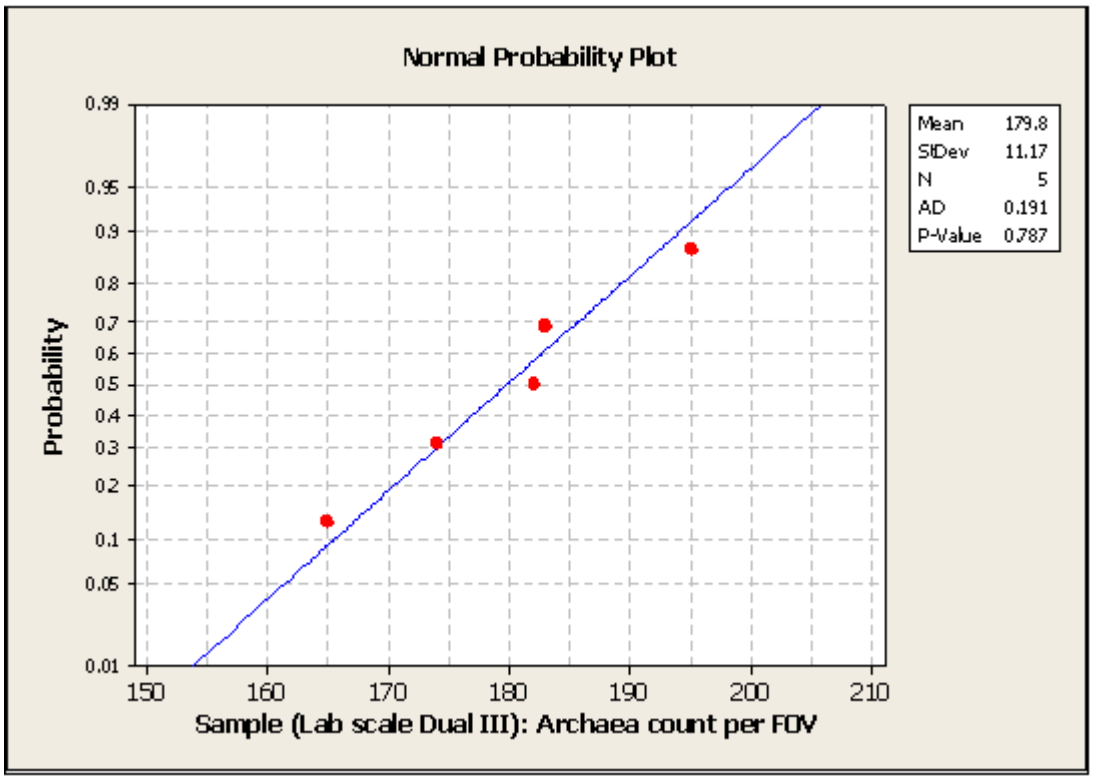
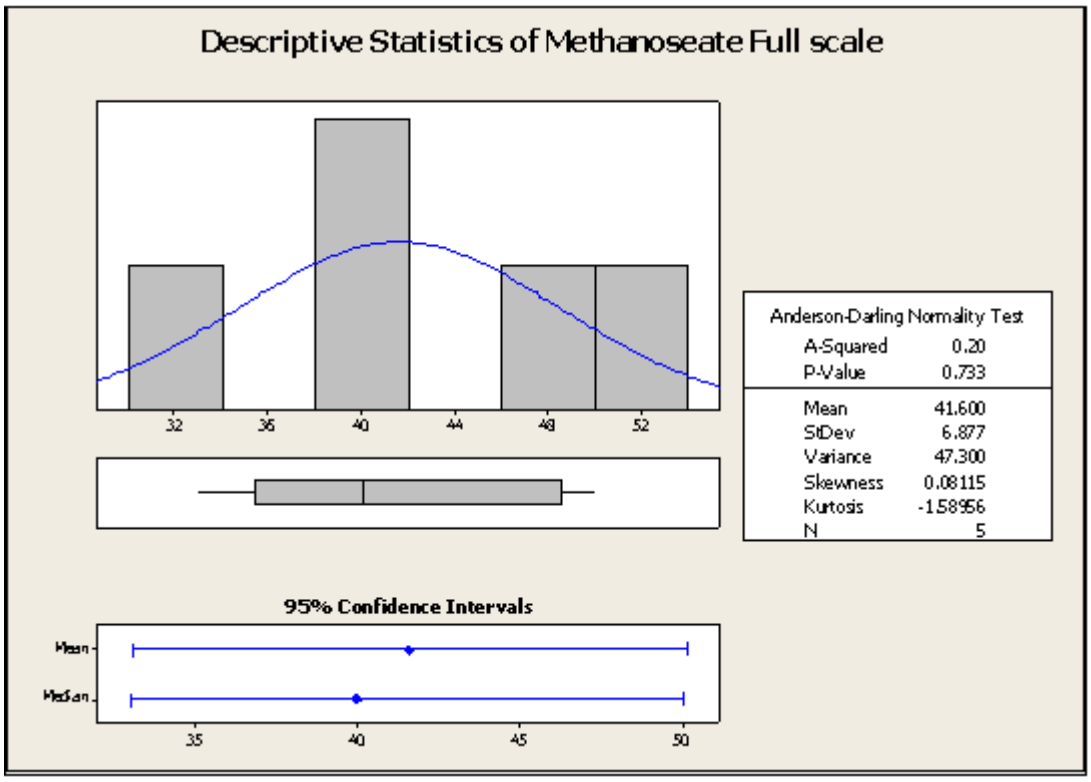
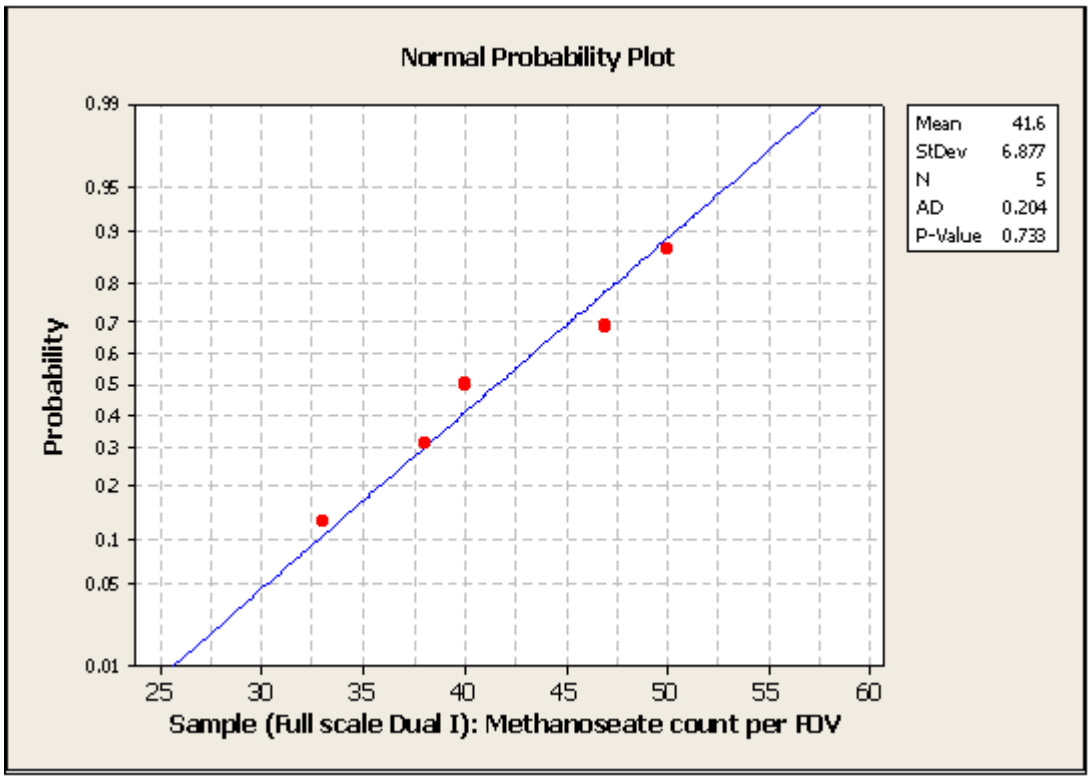
95% Confidence Intervals
Mean
Median

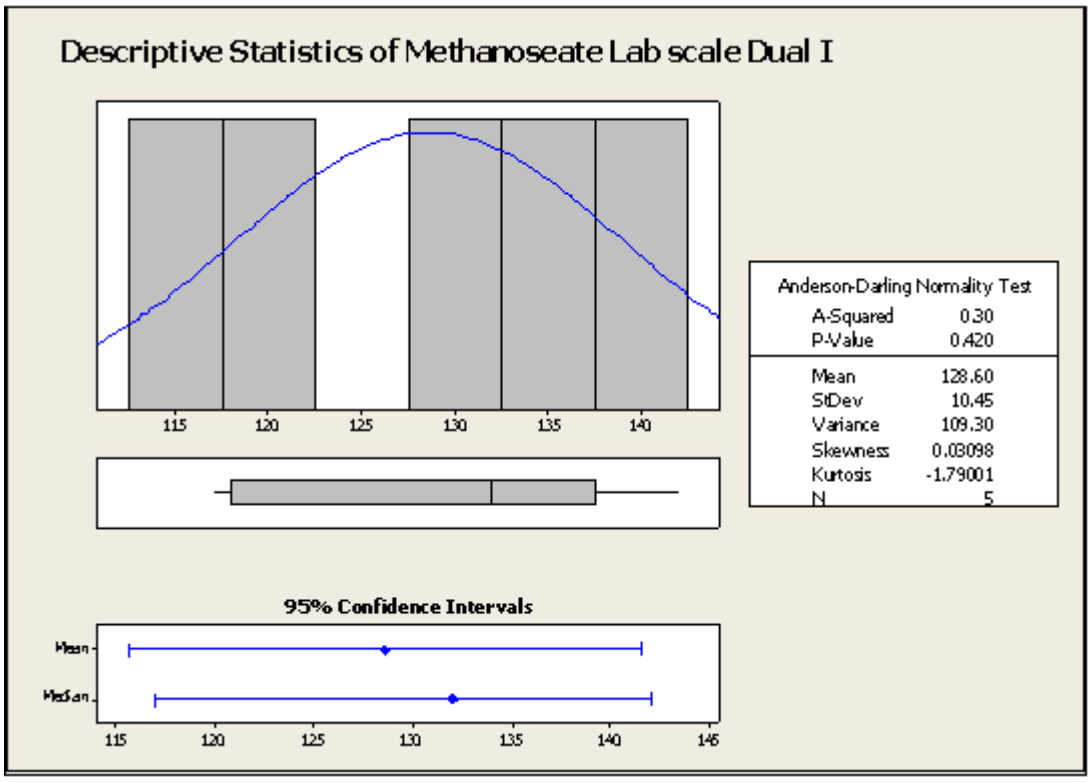

Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
160
170
180
190
200
210
220
230
Sample (Lab scale Dual II): Archaea count per FOV
Mean 193.6
StDev 12.90
N 5
AD 0.297
P-Value 0.430

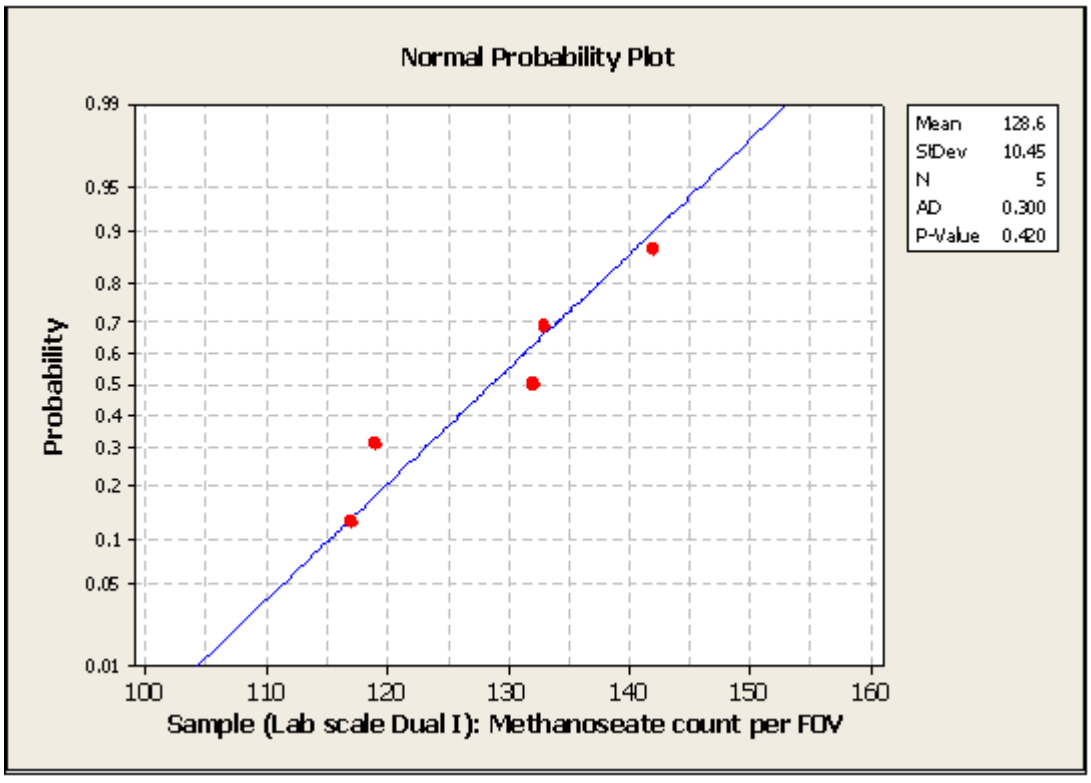

Descriptive Statistics of Archaea Lab scale Dual III
165
170
175
180
185
190
195
Anderson-Darling Normality Test
A-Squared 0.19
P-Value 0.787
Mean 179.80
StDev 11.17
Variance 124.70
Skewness 0.035404
Kurtosis 0.248982
N 5
95% Confidence Intervals
Mean
MeSan
165
170
175
180
185
190
195

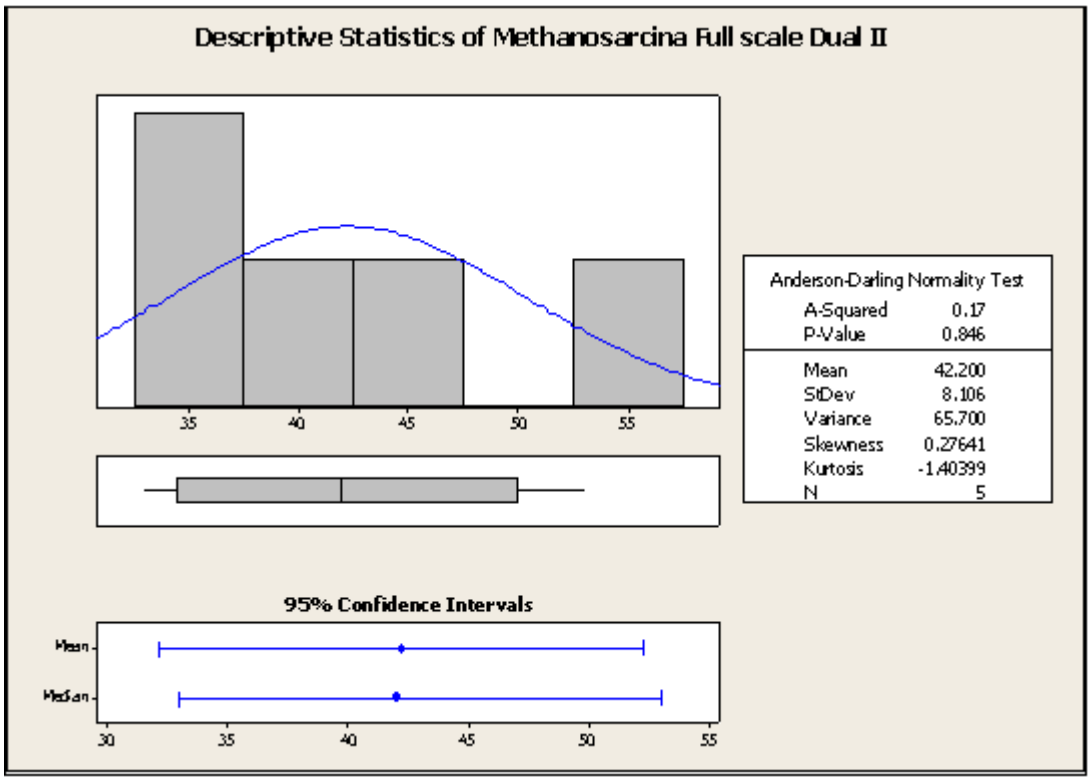

Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
150
160
170
180
190
200
210
Sample (Lab scale Dual III): Archaea count per FOV
Mean 179.8
StDev 11.17
N 5
AD 0.191
P-Value 0.787

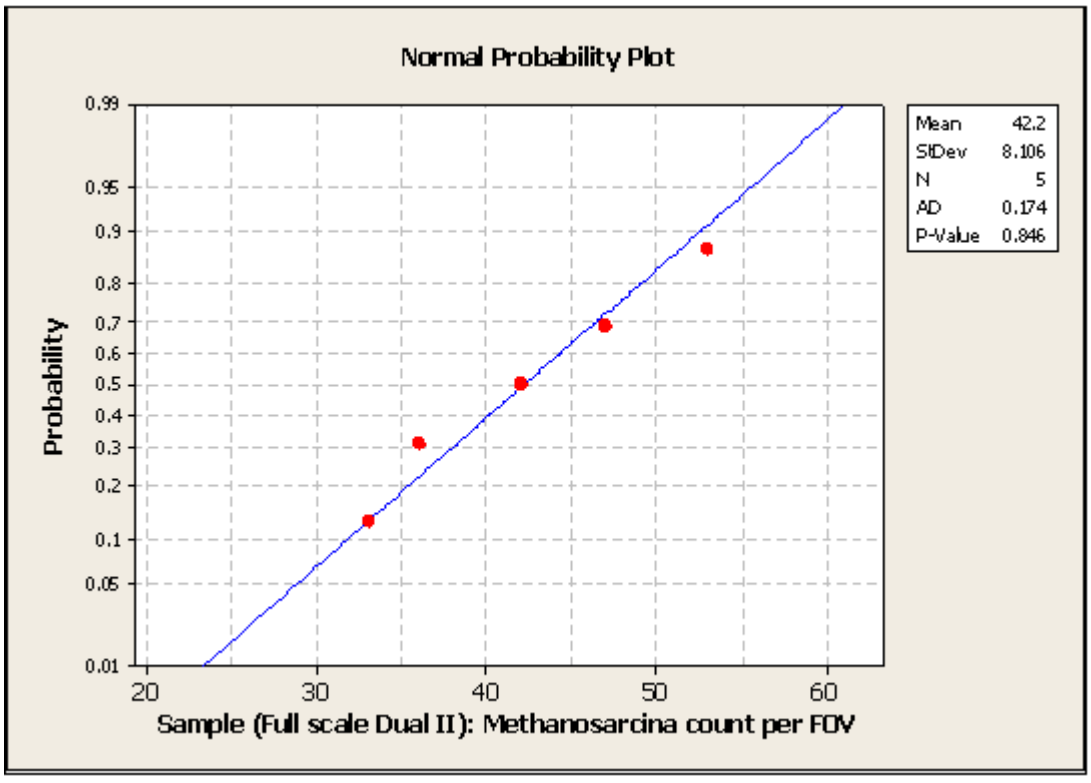

Descriptive Statistics of Methanoseate Full scale
32
36
40
44
48
52
Anderson-Darling Normality Test
A-Squared 0.20
P-Value 0.733
Mean 41.600
StDev 6.877
Variance 47.300
Skewness 0.08115
Kurtosis -1.58956
N 5
95% Confidence Intervals
Mean
Median
35
40
45
50

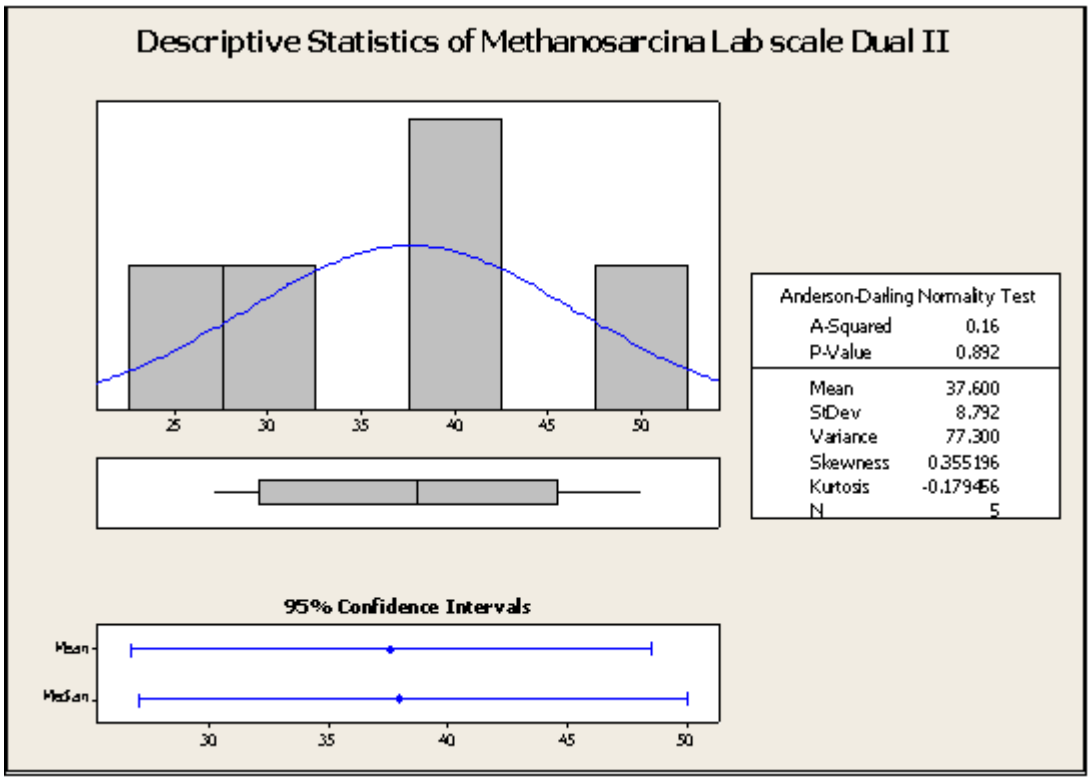

Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
25
30
35
40
45
50
55
60
Sample (Full scale Dual I): Methanoseate count per ROV
Mean 41.6
StDev 6.877
N 5
AD 0.204
P-Value 0.733

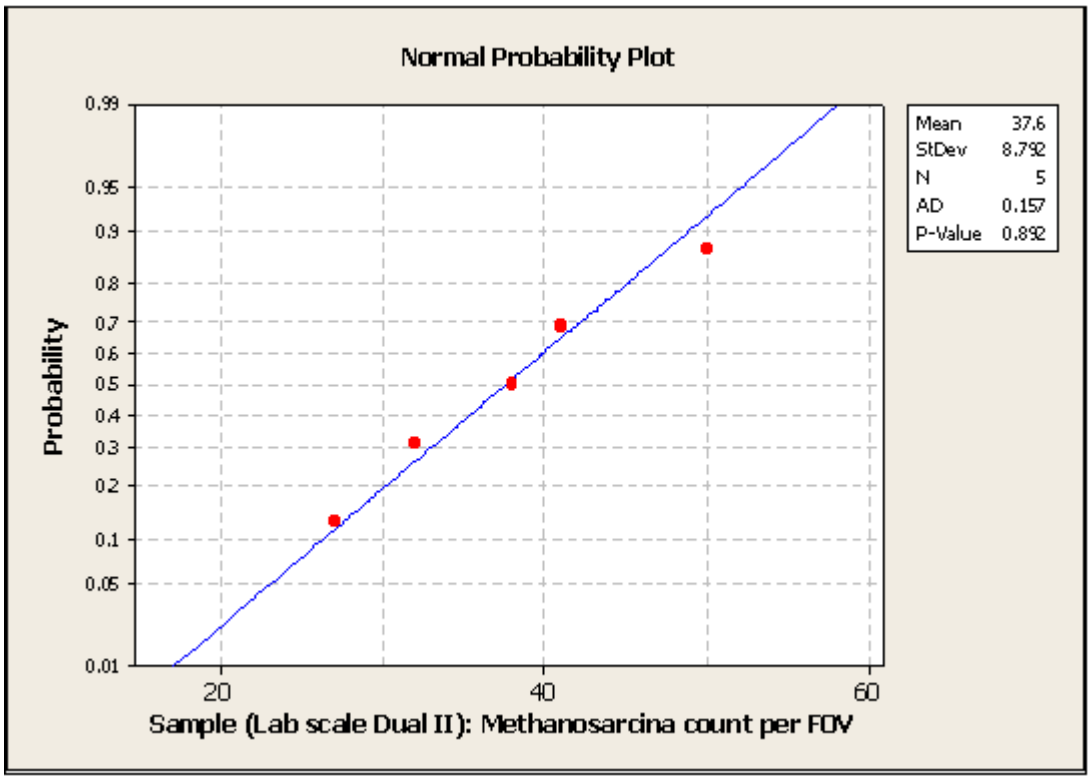

Descriptive Statistics of Methanoseate Lab scale Dual I
115
120
125
130
135
140
Anderson-Darling Normality Test
A-Squared 0.30
P-Value 0.420
Mean 128.60
StDev 10.45
Variance 109.30
Skewness 0.03098
Kurtosis -1.79001
N 5
95% Confidence Intervals
Mean
Median
115
120
125
130
135
140
145

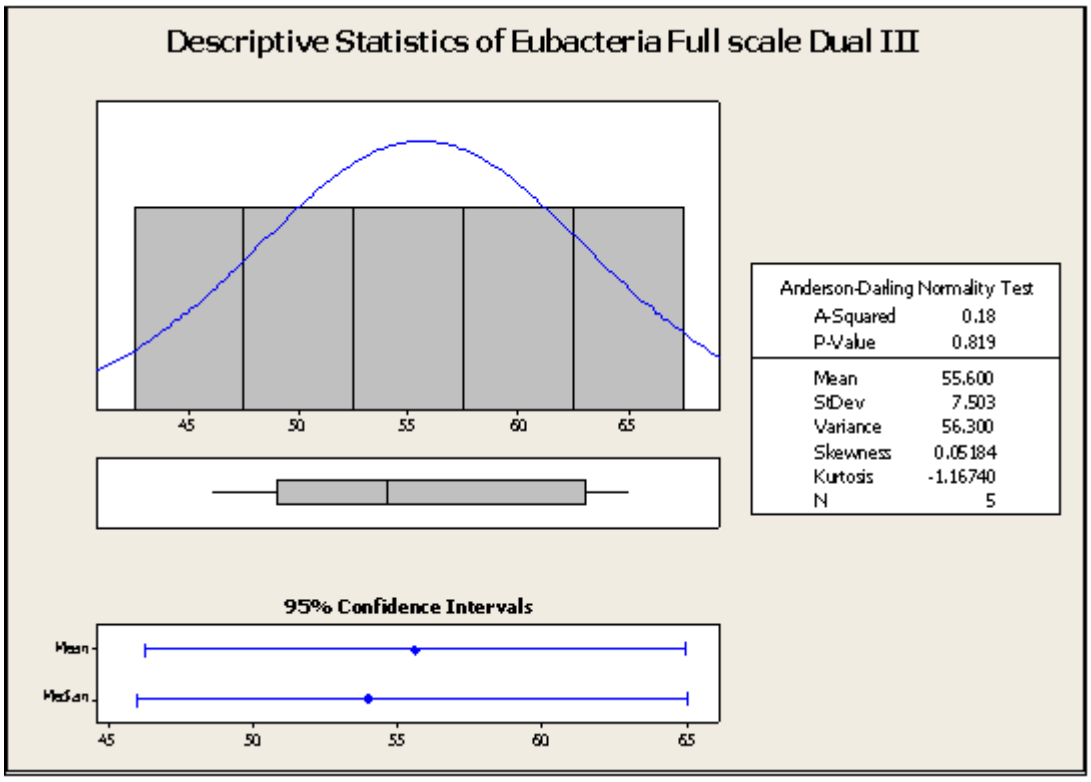

Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
100
110
120
130
140
150
160
Sample (Lab scale Dual I): Methanoseate count per FOV
Mean 128.6
StDev 10.45
N 5
AD 0.300
P-Value 0.420

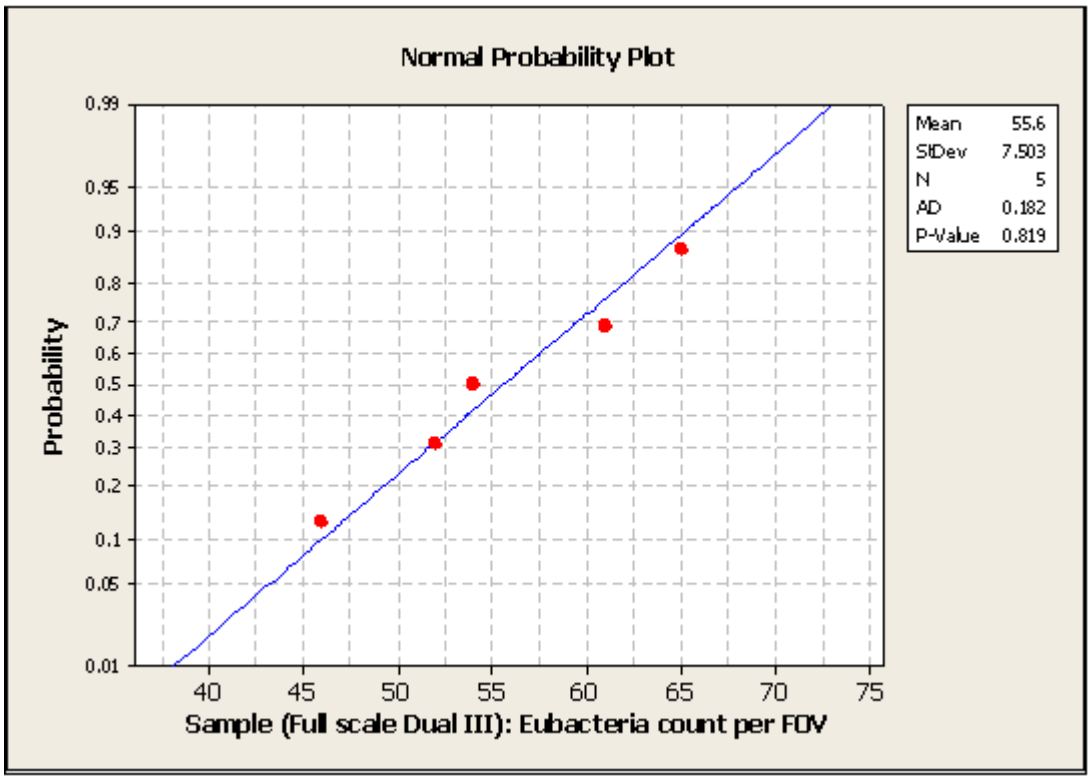

Descriptive Statistics of Methanosarcina Full scale Dual II
35
40
45
50
55
Anderson-Darling Normality Test
A-Squared 0.17
P-Value 0.846
Mean 42.200
StDev 8.106
Variance 65.700
Skewness 0.27641
Kurtosis -1.40399
N 5
95% Confidence Intervals
Mean
Median
30
35
40
45
50
55

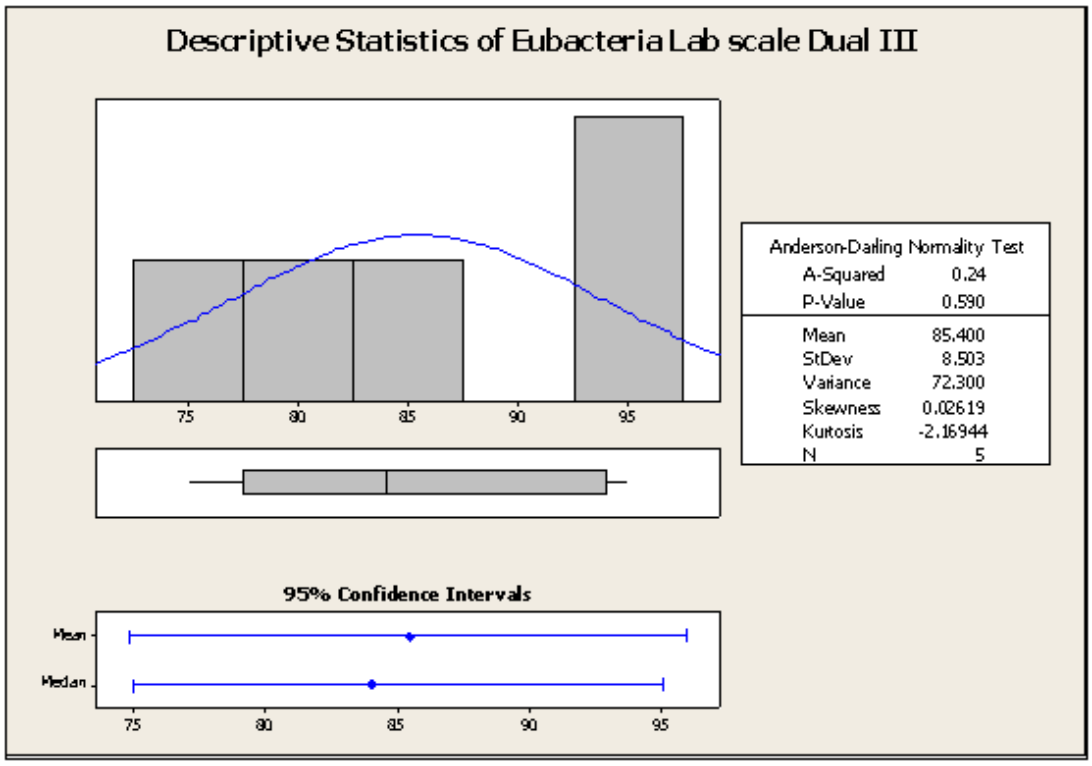

Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
20
30
40
50
60
Sample (Full scale Dual II): Methanosarcina count per FOV
Mean 42.2
StDev 8.106
N 5
AD 0.174
P-Value 0.846


Descriptive Statistics of Methanosarcina Lab scale Dual II
25
30
35
40
45
50
Anderson-Darling Normality Test
A-Squared 0.16
P-Value 0.892
Mean 37.600
StDev 8.792
Variance 77.300
Skewness 0.355196
Kurtosis -0.179456
N 5
95% Confidence Intervals
Mean
Median
30
35
40
45
50


Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
20
40
60
Sample (Lab scale Dual II): Methanosarcina count per FOV
Mean 37.6
StDev 8.792
N 5
AD 0.157
P-Value 0.892


Descriptive Statistics of Eubacteria Full scale Dual III
45
50
55
60
65
Anderson-Darling Normality Test
A-Squared 0.18
P-Value 0.819
Mean 55.600
StDev 7.503
Variance 56.300
Skewness 0.05184
Kurtosis -1.16740
N 5
95% Confidence Intervals
Mean
Median
45
50
55
60
65


Normal Probability Plot
Probability
0.99
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.01
40
45
50
55
60
65
70
75
Sample (Full scale Dual III): Eubacteria count per FOV
Mean 55.6
StDev 7.503
N 5
AD 0.182
P-Value 0.819



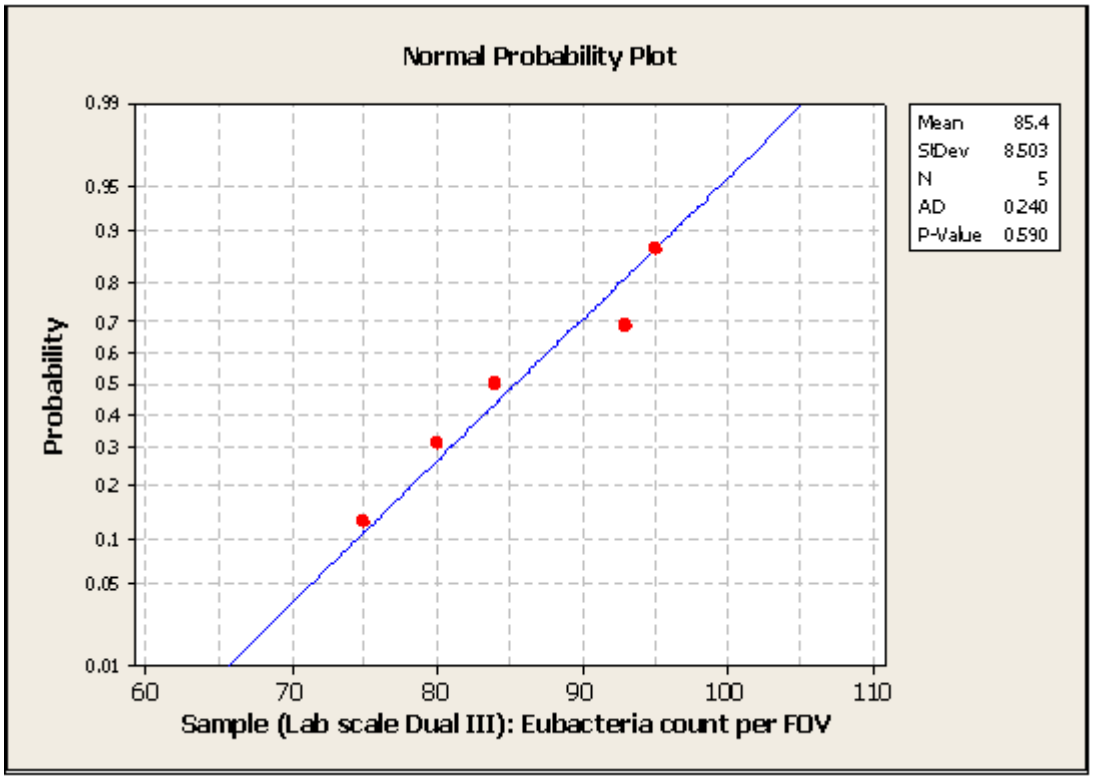
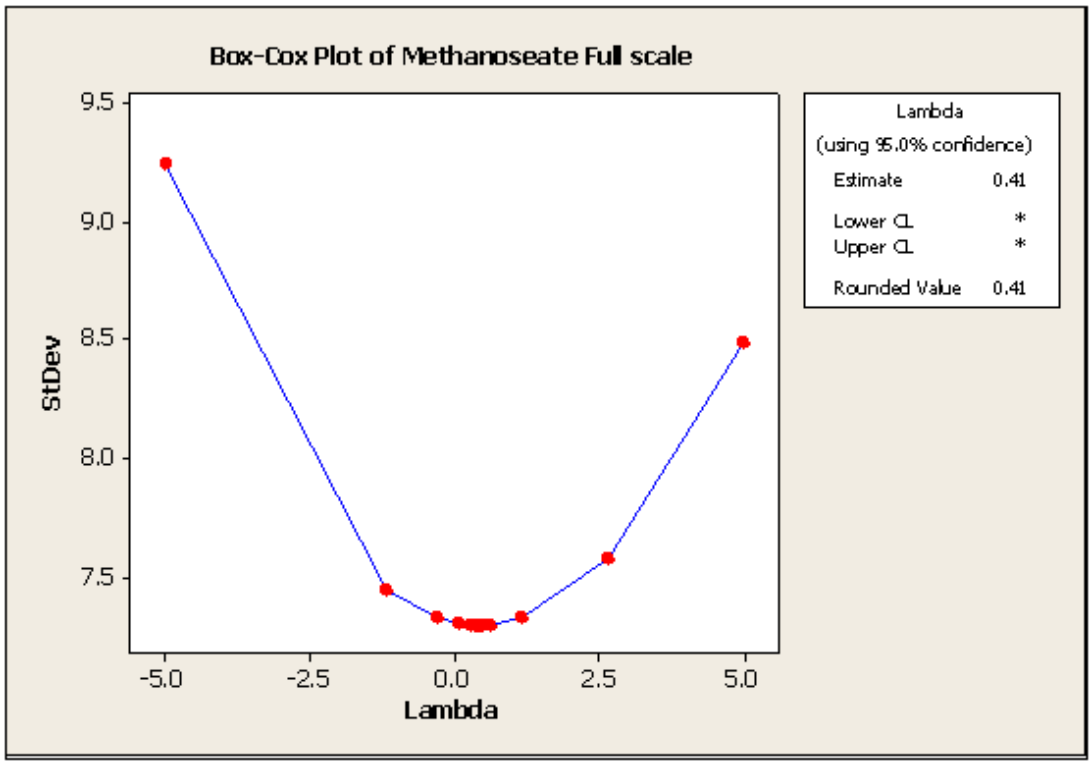
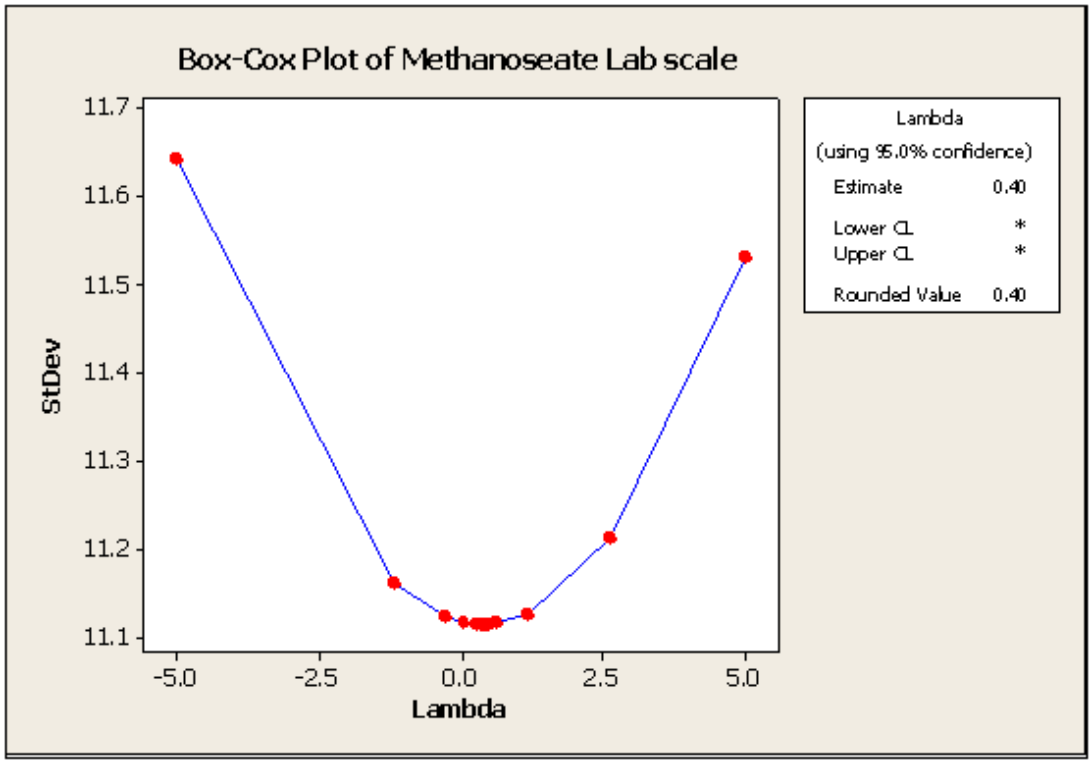
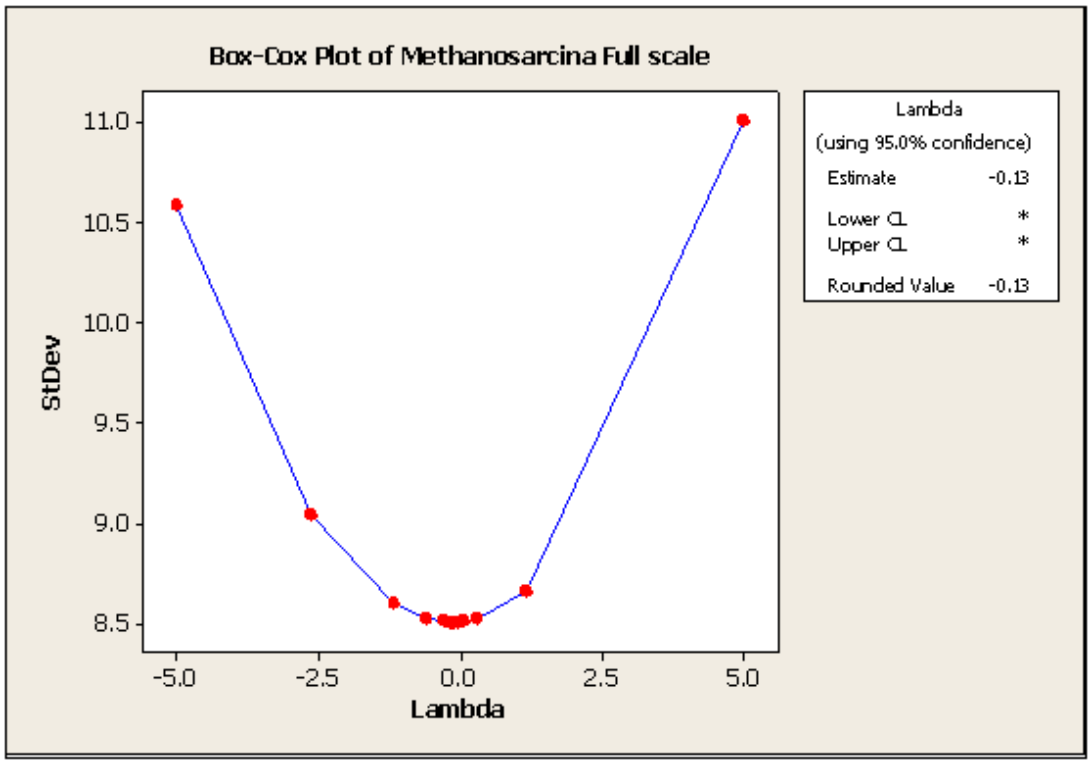
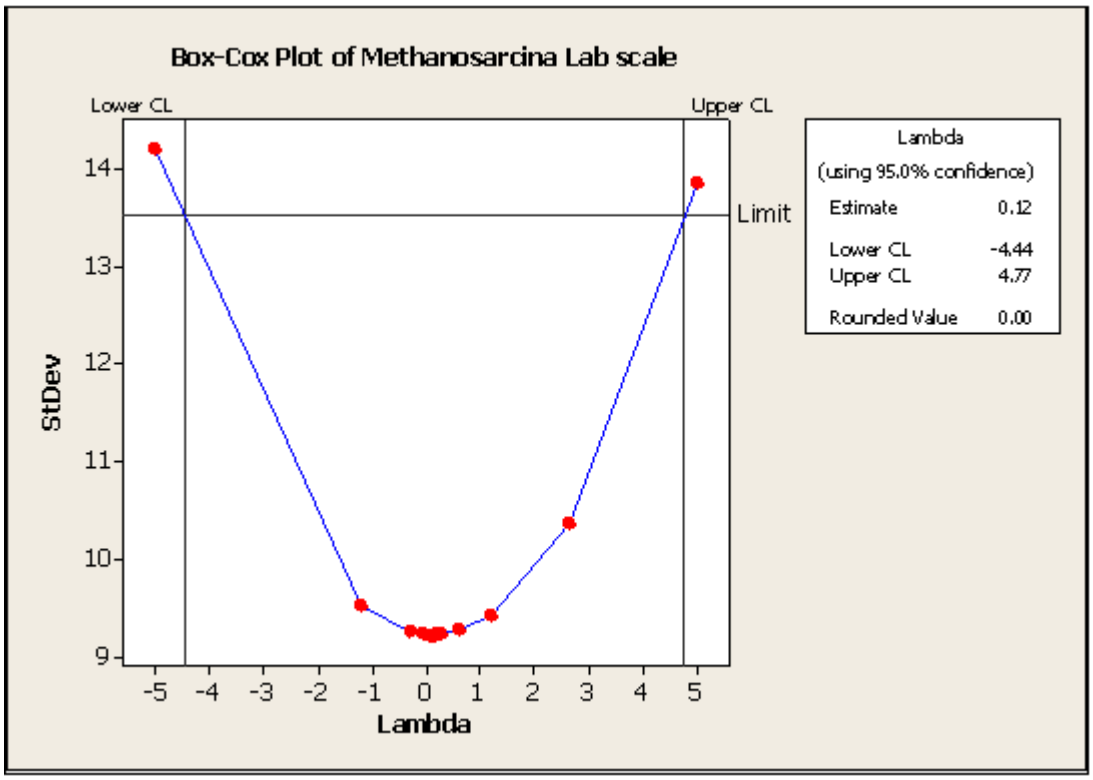


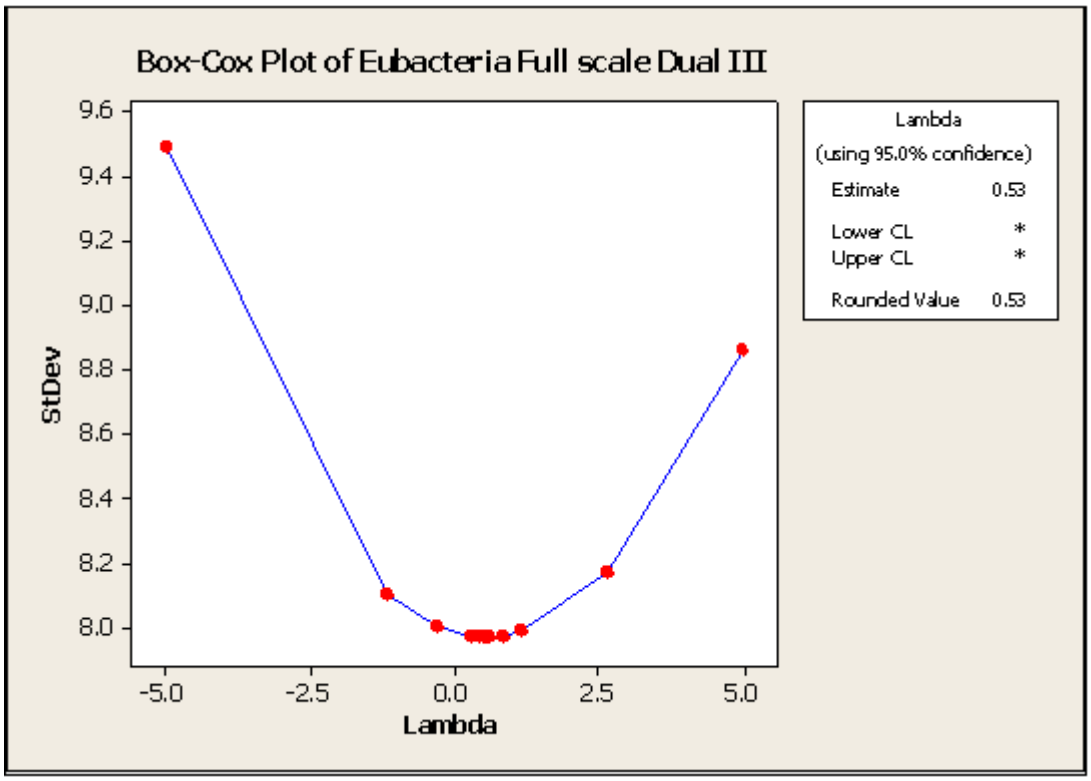
Figure A. MINITAB outputs for frequency distributions with normality curves, descriptive statistics and normal probability distributions of *Archaea*, *Methanoseate*, *Methanosarcina* and *Eubacteria* present in the sludge samples.


Box-Cox Plot of Methanoseate Full scale
StDev
9.5
9.0
8.5
8.0
7.5
-5.0
-2.5
0.0
2.5
5.0
Lambda
Lambda
(using 95.0% confidence)
Estimate 0.41
Lower CL *
Upper CL *
Rounded Value 0.41


Box-Cox Plot of Methanoseate Lab scale
StDev
11.7
11.6
11.5
11.4
11.3
11.2
11.1
-5.0
-2.5
0.0
2.5
5.0
Lambda
Lambda
(using 95.0% confidence)
Estimate 0.40
Lower CL *
Upper CL *
Rounded Value 0.40


Box-Cox Plot of Methanosarcina Full scale
StDev
Lambda
11.0
10.5
10.0
9.5
9.0
8.5
-5.0
-2.5
0.0
2.5
5.0
Lambda
(using 95.0% confidence)
Estimate -0.13
Lower CL *
Upper CL *
Rounded Value -0.13


Box-Cox Plot of Methanosarcina Lab scale
Lower CL
Upper CL
Limit
StDev
Lambda
14
13
12
11
10
9
-5
-4
-3
-2
-1
0
1
2
3
4
5
Lambda
(using 95.0% confidence)
Estimate 0.12
Lower CL -4.44
Upper CL 4.77
Rounded Value 0.00



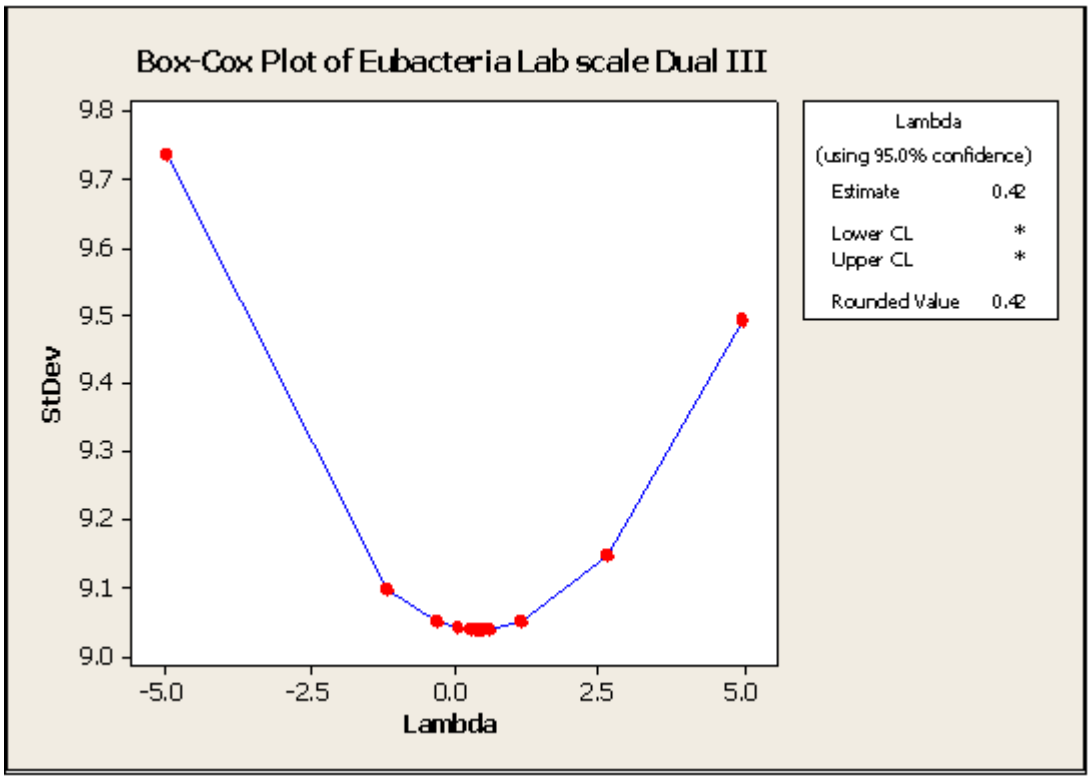


Figure B. The Box-Cox plots of *Methanoseate*, *Methanosarcina* and *Eubacteria* present in the sludge samples taken from the full-scale and lab-scale anaerobic digesters.

Table A. The abundance data and one-way ANOVA analyses of *Methanoseate*, *Methanosarcina* and *Eubacteria* from samples of lab scale and full scale anaerobic digesters following FISH

1a

	Methanoseate in lab scale anaerobic digester			*Methanoseate* in full scale anaerobic digester		
Sampling unit, n (FOV)	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml
1	132	1.82	42717	47	0.81	28448
2	142	1.96	44306	40	0.69	26245
3	119	1.65	40559	38	0.65	25580
4	117	1.62	40217	50	0.86	29342
5	133	1.84	42879	33	0.57	23838
Mean, x^m	-	1.78	**42135**	-	0.72	**26691**
S.D., s	-	0.15	1715	-	0.12	2219
Coefficient of variation, *CV* (%)	-	8.13	4.07	-	16.53	8.31

1b

One-Way Analysis of Variance : SQRT *Methanoseate* conc. Lab-scale, SQRT *Methanoseate* conc. Full scale

```
Source  DF         SS         MS       F      P
Factor   1  596354618  596354618  151.69  0.000
Error    8   31451354    3931419
Total    9  627805972

S = 1983   R-Sq = 94.99%   R-Sq(adj) = 94.36%


                                 Individual 95% CIs For Mean Based on Pooled
                                 StDev
Level          N   Mean  StDev     -+---------+---------+---------+--------
Square root L  5  42135   1715                                   (---*---)
Square root F  5  26691   2219     (---*---)
                                   -+---------+---------+---------+--------
                                  25000     30000     35000     40000

Pooled StDev = 1983


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


Square root L subtracted from:

               Lower  Center   Upper
Square root F  -18337  -15445  -12553

                   -+---------+---------+---------+--------
Square root F      (----*----)
                   -+---------+---------+---------+--------
                 -18000    -12000     -6000         0
```

2a

	Methanosarcina in lab scale anaerobic digester			*Methanosarcina* in full scale anaerobic digester		
Sampling unit, n (FOV)	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	ln number of cells per ml	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	ln number of cells per ml
1	32	0.44	19.9	53	0.91	20.63
2	27	0.37	19.73	42	0.72	20.4
3	41	0.57	20.16	33	0.57	20.16
4	38	0.53	20.08	36	0.62	20.25
5	50	0.69	20.35	47	0.81	20.51
Mean, x^m	-	0.52	**20.05**	-	0.73	**20.39**
S.D., s	-	0.12	0.23	-	0.14	0.19
Coefficient of variation, *CV* (%)	-	23.4	1.18	-	19.2	0.94

2b

One-Way Analysis of Variance : Ln *Methanosarcina* conc. Lab-scale, Ln *Methanosarcina* conc. Full scale

```
Source  DF      SS      MS     F      P
Factor   1  0.2924  0.2924  6.44  0.035
Error    8  0.3633  0.0454
Total    9  0.6557

S = 0.2131   R-Sq = 44.60%   R-Sq(adj) = 37.67%


                                      Individual 95% CIs For Mean Based on
                                      Pooled StDev
Level              N    Mean  StDev  ---------+---------+---------+---------
+
Methanosarcina-L   5  20.048  0.234  (----------*----------)
Methanosarcina-F   5  20.390  0.190                  (----------*---------
)
                                      ---------+---------+---------+---------
+
                                            20.00     20.20     20.40
20.60

Pooled StDev = 0.213


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


Methanosarcina-Lab scale subtracted from:

                   Lower  Center   Upper
Methanosarcina-F  0.0312  0.3420  0.6528

                   --+---------+---------+---------+-------
Methanosarcina-F             (-----------*-----------)
                   --+---------+---------+---------+-------
                  -0.25      0.00      0.25      0.50
```

3a

	Eubacteria in lab scale anaerobic digester			*Eubacteria* in full scale anaerobic digester		
Sampling unit, n (FOV)	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml	Original number of cells per FOV	Number of cells per ml ($\times 10^9$)	SQRT number of cells per ml
1	75	1.04	32199	46	0.79	28144
2	80	1.11	33255	52	0.9	29923
3	93	1.29	35856	61	1.05	32410
4	84	1.16	34077	54	0.93	30493
5	95	1.31	36239	65	1.12	33455
Mean, x^m	-	1.18	**34325**	-	0.96	**30885**
S.D., s	-	0,112	1712,53	-	0,13	2092,93
Coefficient of variation, *CV* (%)	-	9,96	4,99	-	13,5	6,78

3b

One-Way Analysis of Variance : SQRT conc. of *Eubacteria* Lab-scale, SQRT conc. of *Eubacteria* Full scale

```
Source  DF        SS        MS     F      P
Factor   1  29585715  29585715  8.09  0.022
Error    8  29252757   3656595
Total    9  58838472

S = 1912   R-Sq = 50.28%   R-Sq(adj) = 44.07%

                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level             N   Mean  StDev  -----+---------+---------+---------+----
SQRT cons of Eub  5  34325   1713                  (---------*---------)
SQRT cons of Eub  5  30885   2093  (---------*---------)
                                    -----+---------+---------+---------+----
                                     30000     32000     34000     36000

Pooled StDev = 1912


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%


SQRT cons of Eubacteria L subtracted from:

                  Lower  Center  Upper
SQRT cons of Eub  -6229   -3440   -651

                  -----+---------+---------+---------+----
SQRT cons of Eub  (----------*----------)
                  -----+---------+---------+---------+----
                   -5000     -2500         0      2500
```

Table B. Critical values of F_{max}

ν \ a	2	3	4	5	6	7	8	9	10	11	12
2	39.0 199.	87.5 448.	142. 729.	202. 1036.	266. 1362.	333. 1705.	403. 2063.	475. 2432.	550. 2813.	626. 3204.	704. 3605.
3	15.4 47.5	27.8 85.	39.2 120.	50.7 151.	62.0 184.	72.9 21(6)	83.5 24(9)	93.9 28(1)	104. 31(0)	114. 33(7)	124. 36(1)
4	9.60 23.2	15.5 37.	20.6 49.	25.2 59.	29.5 69.	33.6 79.	37.5 89.	41.1 97.	44.6 106.	48.0 113.	51.4 120.
5	7.15 14.9	10.8 22.	13.7 28.	16.3 33.	18.7 38.	20.8 42.	22.9 46.	24.7 50.	26.5 54.	28.2 57.	29.9 60.
6	5.82 11.1	8.38 15.5	10.4 19.1	12.1 22.	13.7 25.	15.0 27.	16.3 30.	17.5 32.	18.6 34.	19.7 36.	20.7 37.
7	4.99 8.89	6.94 12.1	8.44 14.5	9.70 16.5	10.8 18.4	11.8 20.	12.7 22.	13.5 23.	14.3 24.	15.1 26.	15.8 27.
8	4.43 7.50	6.00 9.9	7.18 11.7	8.12 13.2	9.03 14.5	9.78 15.8	10.5 16.9	11.1 17.9	11.7 18.9	12.2 19.8	12.7 21.
9	4.03 6.54	5.34 8.5	6.31 9.9	7.11 11.1	7.80 12.1	8.41 13.1	8.95 13.9	9.45 14.7	9.91 15.3	10.3 16.0	10.7 16.6
10	3.72 5.85	4.85 7.4	5.67 8.6	6.34 9.6	6.92 10.4	7.42 11.1	7.87 11.8	8.28 12.4	8.66 12.9	9.01 13.4	9.34 13.9
12	3.28 4.91	4.16 6.1	4.79 6.9	5.30 7.6	5.72 8.2	6.09 8.7	6.42 9.1	6.72 9.5	7.00 9.9	7.25 10.2	7.48 10.6
15	2.86 4.07	3.54 4.9	4.01 5.5	4.37 6.0	4.68 6.4	4.95 6.7	5.19 7.1	5.40 7.3	5.59 7.5	5.77 7.8	5.93 8.0
20	2.46 3.32	2.95 3.8	3.29 4.3	3.54 4.6	3.76 4.9	3.94 5.1	4.10 5.3	4.24 5.5	4.37 5.6	4.49 5.8	4.59 5.9
30	2.07 2.63	2.40 3.0	2.61 3.3	2.78 3.4	2.91 3.6	3.02 3.7	3.12 3.8	3.21 3.9	3.29 4.0	3.36 4.1	3.39 4.2
60	1.67 1.96	1.85 2.2	1.96 2.3	2.04 2.4	2.11 2.4	2.17 2.5	2.22 2.5	2.26 2.6	2.30 2.6	2.33 2.7	2.36 2.7
∞	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00	1.00 1.00

CURRICULUM VITAE

Meriç BATIOĞLU was born in Edirne, 1982. She graduated from Yeşilevler Elementary School and Mimar Sinan Foreign Language High School. Her first degree is Biology in İstanbul University. After her graduation, she has been accepted to the Environmental Biotechnology M.Sc. programme in İstanbul Technical University. After one year, she continued her postgraduate study in University of Newcastle upon Tyne (the United Kingdom) and finished her research project with distinction grade.